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THE SUBCELLULAR FRACTIONATION AND PURIFICATION
OF INTRACELLULAR CYSTEINE PROTEINASES IN VEGETATIVE AMOEBAE
OF THE CELLULAR SLIME MOLD, DICTYOSTELIUM DISCOIDEUM

By

Chelong Wang

B.S., Fu-Jen Catholic University, 1982

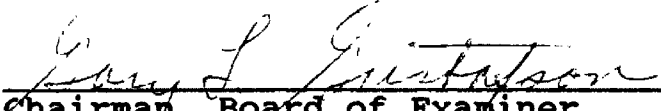
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degree of

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The Subcellular Fractionation and Purification of Intracellular Cysteine Proteinases in Vegetative Amoebae of the Cellular Slime Mold, Dictyostelium discoideum. (88 pp.)

Director: Gary L. Gustafson

G.L.G.

It was shown that the cellular slime mold, D. discoideum., produced three cysteine proteinases that had different intracellular compartmentations but shared a common, phosphoglycosylated subunit. In this thesis, the subcellular fractionation was based on a differential centrifugation and the differences in phosphoryl contents of each cysteine proteinase was reflected by differences in their potentials to precipitate with protamine sulfate.

Two of the proteinases were recovered from the cytosolic fraction of cell homogenates, while the third proteinase was recovered from the vacuolar fraction. The proteinases formed stable complexes with a proteinase inhibitor from D. discoideum. Each proteinase and respective proteinase-inhibitor complex migrated as a unique protein band during non-dissociating polyacrylamide gel electrophoresis. Immunoblot analyses showed that the common subunit in the proteinases contained phosphodiester-linked N-acetylglucosamine-1-phosphate groups. Fractionation studies suggested that the cytosolic proteinases occurred as proteinase-inhibitor complexes in intact cells. In contrast, the vacuolar proteinase apparently occurred only in a free state in intact cells.

According to the results of trichloroacetate treatment, cytosolic proteinases could degrade inhibitor in 1.3 M of sodium trichloroacetate at pH 5.5. The inhibitor free cytosolic proteinases were purified after the trichloroacetate treatment.

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INTRODUCTION

1. PROTEINASE

A. General introduction

Proteolytic enzymes (proteases or proteinases) were among the first enzymes recognized. Traditionally the proteinases have been regarded as degradative enzymes which are capable of cleaving proteins into small peptides and amino acids. During the last decade there have been substantial advances in our understanding of cellular processes involving proteolysis, including intracellular protein synthesis, protein turnover, protein degradation, protein translocation, enzyme activation, hormone action and inactivation, blood coagulation, complement system, mammalian reproduction, inflammation and wound healing, sporulation, and germination (1, 2, 3).

Proteolysis may play a number of roles in pathogenesis. For example, many eucaryotic microorganisms need proteinases to penetrate the host organism and to counter host defense mechanisms during infection (2), and some tumor cells need proteinases to invade (3) and to metastasize (4). Analyses of proteolytic enzymes may provide insights about the pathogenesis and methods for the control of disease (2).

Studies of proteolytic enzymes are also merited because of the importance of these enzymes as reagents in laboratory, clinical, and industrial processes. Proteinases are in

widespread use in the food industries (eg; baking, brewing, cheese manufacturing and meat tenderizing), in the tanning industry, and in the manufacture of biological detergents (3).

B. Classification

Early investigators tended to name proteinases according to their origin or apparent function; they were classified in a number of ways. For example, on the basis of pH optima, on the basis of their abilities to hydrolyze specific proteins (elastase, collagenase, etc), or on the basis of their similarities to well-characterized proteinases such as papain, trypsin, or the mammalian cathepsins.

More recently, a more satisfactory classification system has gained preference. It classifies proteinases on the basis of similarities in their enzymatic mechanisms. There are four different types of proteinases, serine, cysteine, aspartic, and metallo proteinases, and these can be distinguished from one another on the basis of their sensitivities to various inhibitors (Table 1).

TABLE 1. Proteinase Classification.

Type	Identification		Examples
	Figure	Inhibitor	
Serine	Active Serine	Fluorophosphate PMSF TLCK	Trypsine Plasmin Subtilisin
Cysteine	CySH	Iodoacetate Iodoacetamide TLCK cystatin	Papain Cathepsin B Proteinase 1
Aspartic	Acidic pH	Diazoketones Pepstatin	Pepsin Chymosin
Metallo	Zn ⁺⁺ , Ca ⁺⁺	Chelating agents EDTA O-phenanthroline	Thermolysin

2.CYSTEINE PROTEINASE

The cysteine proteinases (also called thiol proteinases) contain essential cysteine residues that are involved in covalent intermediate complex with substrates. Most of the cysteine proteinases have non-specific proteolytic activity. They may be involved with the initial or terminal stages of extensive degradation of proteins. Some cysteine proteinases from higher organisms have been well-characterized. Plant enzymes such as papain, ficin, and bromelain are widely used as laboratory and industrial reagents (5). In mammalian cells many of the lysosomal proteinases are of the cysteine type, eg; Cathepsin B, H and L; and they play important roles in general protein degradation. Only two bacterial cysteine proteinases, clostripain and streptococcal proteinase, have been found. However, there are two groups of eucaryotic microorganisms in which highly active cysteine proteinases have been found, protozoa and cellular slime molds (7).

3.PROTEINASES IN DICTYOSTELIUM DISCOIDEUM

A. Biology of Dictyostelium discoideum

Dictyostelium discoideum is a species of cellular slime mold. It was first described in 1935 when it was found in soils from deciduous woods in North Carolina, U. S. A. (33). Since then the life cycle of this microorganism has been much studied. Over the last 20 years, D. dicsoideum has been an attractive model system for biochemists studying fundamental

aspects of the molecular regulation of cellular differentiation processes.

In the laboratory, wild type strains of D. discoideum, can be conveniently co-cultivated with E. coli or other Gram negative bacteria with conventional bacteriological media. Axenic mutants, which can utilize dissolved nutrients in place of bacteria, have also become useful in studies of molecular regulation (6).

B. Proteinases in Dictyostelium discoideum

At least 8 acid proteinases of varying electrophoretic mobility have been observed in extracts of amoebae of D. discoideum (7-12), and these include both aspartic and cysteine proteinases (7,12). The activities attributable to these enzymes are found predominantly in a vacuolar fraction (15,000 xg pellet) (9,13), and they are almost certainly lysosomal proteinases with properties similar to the aspartic and cysteine cathepsins of mammalian cells. Two exopeptidase activities have been reported in cytosolic fraction (15,000 xg supernatant) (9). They include leucine aminopeptidase (14) and alanine aminopeptidase (15).

C. Cysteine proteinases in Dictyostelium discoideum

Two cysteine proteinases have been purified from D. discoideum, Proteinase 1 (12) and Proteinase B (11). The isolation and characterization of proteinase 1 from D. discoideum was first reported by Gustafson and Thon (12). who proposed that the proteinase might play a role in catalyzing the rapid intracellular turnover of UDP-glucose pyrophosphorylase that occurs during the initial hours of differentiation of the slime mold. Proteinase 1 constitutes about 2% of total protein of wild type amoebae. The esterase activity towards α -N-carbo-benzoxymethyl-L-lysine-p-nitrophenol (CBZ-Lys-ONp) ester (pH optimum 5.5) was used for routine assays of this enzyme. It was activated by dithiothreitol and inactivated by iodoacetamide, cystamine, and tosyl-lysine chloromethyl ketone (TLCK). Subsequently chemical analyses and NMR studies showed that proteinase 1 contained multiple N-acetyl-glucosamine-1-phosphate (GlcNAc-P) residues linked through phosphodiester bonds to peptidyl serine (16,17,18).

4. PROTEINASE INHIBITOR

A. General introduction

Proteinase inhibitors have been isolated from a variety of plants, microorganisms, and animal tissues. The most obvious function of proteinase inhibitors would appear to be prevention of inappropriate proteolytic activity. Intracellular inhibitors may function to prevent inappropriate

proteolysis by lysosomal enzymes released into the cytoplasm of a cell under stress conditions. It has also been suggested that inhibitors may play a role in the regulation of proteinase activity. In some cases, fluctuations in cellular proteinase activity may be a result of changes in inhibitor, rather than proteinase levels, concentration (21).

B. Classification

In general, the proteinase inhibitors are classified according to the class of proteinases which they inhibit. There are four major classes - inhibitors of aspartic, metallo, serine and cysteine proteinases. Cysteine proteinase inhibitors of animals are called, "cystatins". They have been found in chicken egg white (21), in rat liver (22), in rabbits (23), human skin (24), and in a variety of other tissues (21).

C. Cystatin in Dictyostelium discoideum

Recently, a cysteine proteinase inhibitor was purified from D. discoideum (19,20). Subcellular fractionation of D. discoideum amoebae revealed that this heat-stable inhibitor was located in the cytosolic fraction. It was suggested that the function of this inhibitor was to block the proteolytic activity of lysosomal enzymes that might be inadvertently transferred to the cytoplasm of cells during stress.

5.OBJECTIVES OF THESIS PROJECT

In extending the studies of the above investigations, I have obtained evidence for the occurrence of cytosolic proteinase-inhibitor complexes in D. discoideum, and have developed several methods for studying these complexes. Based on these preliminary studies I proposed to characterize the cytosolic proteinase-inhibitor complexes and to compare their constituent proteinases with the lysosomal proteinases previously isolated from D. discoideum. The specific aims were as follows:

- a) Cell fractionation procedures were developed for resolving cytosolic and vacuolar proteinases.
- b) The phosphorylated cytosolic and vacuolar proteinases that were precipitated by protamine sulfate were purified.
- c) Methods were developed for dissociating cytosolic proteinase-inhibitor complexes such that unmasked cytosolic proteinase could be purified.
- d) The unmasked cytosolic proteinases were purified.
- e) The structure properties of vacuolar and cytosolic proteinases were compared with regards to 1) their structural properties, 2) their contents of the N-acetyl-glucosamine-1-phosphate (GlcNAc-P-Ser) modification.
- f) The regulation of proteinases and inhibitor levels during differentiation and heat stress response was examined.

- g) The relative levels of the cytosolic and vacuolar proteinases produced by axenically cultured cells were compared with the proteinases levels observed in cells grown on bacteria.

MATERIALS AND METHODS

MATERIALS

Centrifuges used were as follows: Sorvall RC 2-B, Beckman J-6B centrifuge and Beckman L2-65B ultracentrifuge. A Coleman Junior 2 spectrophotometer was used in the determination of protein. The Perkin-Elmer Lambda-3 spectrophotometer system was used in the assay of proteinase activity. Sephadex G-200 was from Pharmacia. DEAE-Cellulose, type 52, was from Whatman. Pressure Ultrafiltration equipment was from Amicon. Dialysis tubing (3500 MW cut off) was from Spectrum Medical Industries. Nitrocellulose membranes for electroblotting (0.45 μ pore size) were from Microfiltration systems. Peptone and Noble agar were purchased from Difco Laboratories and yeast extract was obtained from BBL. Stainsall (3,3'-diethyl-9-methyl-4,5,4',5'-dibenzothiacarbo-cyanine), tricine, TLCK (Tosyl-lysine chloromethy ketone), DTT (Dithiothreitol) and CBZ-Lys-ONp (α -N-carbo-benzoxy-L-lysine-p-nitrophenol) were purchased from Sigma Chemical Co. Acrylamide, bisacrylamide, and guanidine HCl were from United States Biochemical Corp. Goat-anti-rabbit horseraddish peroxidase conjugate was from BioRad. All other chemicals were reagent grade.

The following buffers were employed. Buffer A: 0.5 M sucrose, 1 mM MES/NaOH, pH 6.0. buffer B: 10 mM tricine/HCl, 1 mM DTT, pH 8.0. Buffer C: 10 mM tricine/HCl, 0.3 M NaCl, 1 mM DTT, pH 8.0.

Buffer D: 40 mM potassium phosphate, pH 6.5. Buffer E: Standard esterase activity assay buffer, 0.2 M sodium acetate, pH 5.5. Buffer F: 0.2 M sodium acetate, 1.3 M trichloroacetate, pH 5.5. HL-5 medium: peptone, 15 g; yeast extract, 7.5 g; glucose, 15 g; streptomycin sulfate, 250 mg.

The antisera used in these studies were prepared as follows:

- 1) Anti-Pr1 Anti-Pr1 was a polyclonal antiserum generated against the purified Proteinase 1 which consisted of a mixture of free Proteinase 1 and inhibitor complex (26).
- 2) Anti-Pr1-HF Anti-Pr1-HF was a polyclonal antiserum generated against the chemically dephosphorylated Proteinase 1. Antibodies in this antisera were judged to be specific for the peptide structure of Proteinase 1 (26).
- 3) Anti-Pr1-N-Acetylglucosamine-1-Phosphate Affinity Purified Antibodies against the N-acetyl-glucosamine-1-phosphate moiety of Proteinase 1 (27) were isolated from polyclonal anti-Pr1. These antibodies were purified on an affinity column 'conjugated with Uridine Diphosphate-N-Acetylglucosamine (UDP-GlcNAc).
- 4) Anti-I Anti-I was an antiserum generated against the purified proteinase inhibitor (20).

METHODS

1. CELL CULTURE CONDITION

The wild type strain of D. discoideum, NC-4, was grown in association with E. coli, and the axenic strain, Ax-3, was grown axenically.

A. Preparation and storage of spore plate

Stock cultures of D. discoideum were maintained as fruiting bodies on nutrient agar plates stored at 4°C. New spore plates were prepared every 4 weeks so as to maintain fresh stock cultures of the organism. Nutrient agar used in preparing spore plates contained in 550 ml; yeast, 0.5 g; peptone, 5 g; glucose, 5 g; K₂HPO₄, 0.48 g; KH₂PO₄, 0.73 g; MgSO₂, 0.5 g and agar, 10 g. The medium was sterilized for 20 minutes at 121°C, and poured into petri dishes. The nutrient agar plates were inoculated with 1) 0.5 ml of E. coli culture containing 10⁸ -10⁹ cells/ml; and 2) 5 - 10 spore heads from slime mold fruiting bodies (derived from a spore plate prepared previously). The mixture of bacteria and spores were incubated at 23°C for 3 days.

B. Growth of cells in association with bacteria

Cells growing in association with E. coli were cultured in covered aluminum pans (16 x 11 x 1 inches). Each pan contained 550 ml of sterile nutrient agar medium (same

composition as used for agar plates). The pans were inoculated with 4 ml of a suspension of *E. coli* cells and slime mold spores, prepared by mixing the spores from one spore plate with 100 ml of an *E. coli* culture (10^8 - 10^9 cells/ml). The pans were incubated at 23°C. At designated time intervals, amoebae were harvested from the pan in cold Buffer A, and washed by repeated centrifugations 3 times at 1,000 xg, 1 minute each, in order to remove bacteria. Washed amoebae were resuspended in Buffer A at a density of 1.5×10^9 cells/ml and fractionated as described in Section 3. All steps were performed at 4°C.

C. Growth of cells on HL-5 medium

A stock culture of Ax-3 strain cells was incubated in fresh HL-5 medium. The culture was incubated at 23°C and shaken at 120 rpm. Aliquots of cell cultures were collected at designated time intervals or at designated cell densities and sedimented by centrifugation at 750 xg for 1 minute. The cell pellets were washed 3 times with cold Buffer A. Washed cells were resuspended in Buffer A at a cell density of 1×10^5 cells/ml and fractionated as described in Section 3.

D. Differentiation of cells

In experiments requiring differentiated cells, cells were treated as follows: a) stationary phase amoebae were collected after most of the bacteria were depleted but prior to the start of cellular aggregation; b) amoebae were washed free of

bacteria by repeated centrifugation in cold distilled water, and resuspended in Buffer D at a density of 1×10^8 cells/ml; c) 0.8 ml of the cell suspension was spread onto each of several black nitrocellulose membrane circles supported on absorbent pads containing 1.2 ml Buffer D; and d) the membrane-pad systems were incubated in a moist environment at 23°C. The extracellular fluid was collected from absorbent pads by centrifugation after 16 hours of differentiation.

E. Heat stress of cells

Vegetative amoebae that had been cultured to stationary phase at 23°C on nutrient agar were transferred to an incubator at 40°C. At designated time intervals the cells were harvested in Buffer A and washed by repeated centrifugation.

2. ASSAY FOR ESTERASE ACTIVITY

Esterase activity was measured using a Perkin-Elmer, Lambda 3 Spectrophotometer.

A. Assays employing Buffer E

Unmasked esterase activity was assayed in Buffer E. Between 0.010 and 0.050 ml of proteinase sample was mixed with Buffer E to give a total volume of 0.940 ml in each 1 ml cuvette. DTT was then added to the cuvettes (0.010 ml of a 100 mM preparation), and reactions were initiated by addition of 0.050 ml of substrate (5 mM CBZ-Lys-ONp in 50% acetonitrile).

The activity was measured at 340 nm against a proteinase-free substrate blank. One unit of esterase activity was defined as that amount which catalyzed liberation of one micromole of p-nitrophenol per minute (extinction coefficient, 5600 M^{-1} at pH 5.5).

B. Assay employing Buffer F

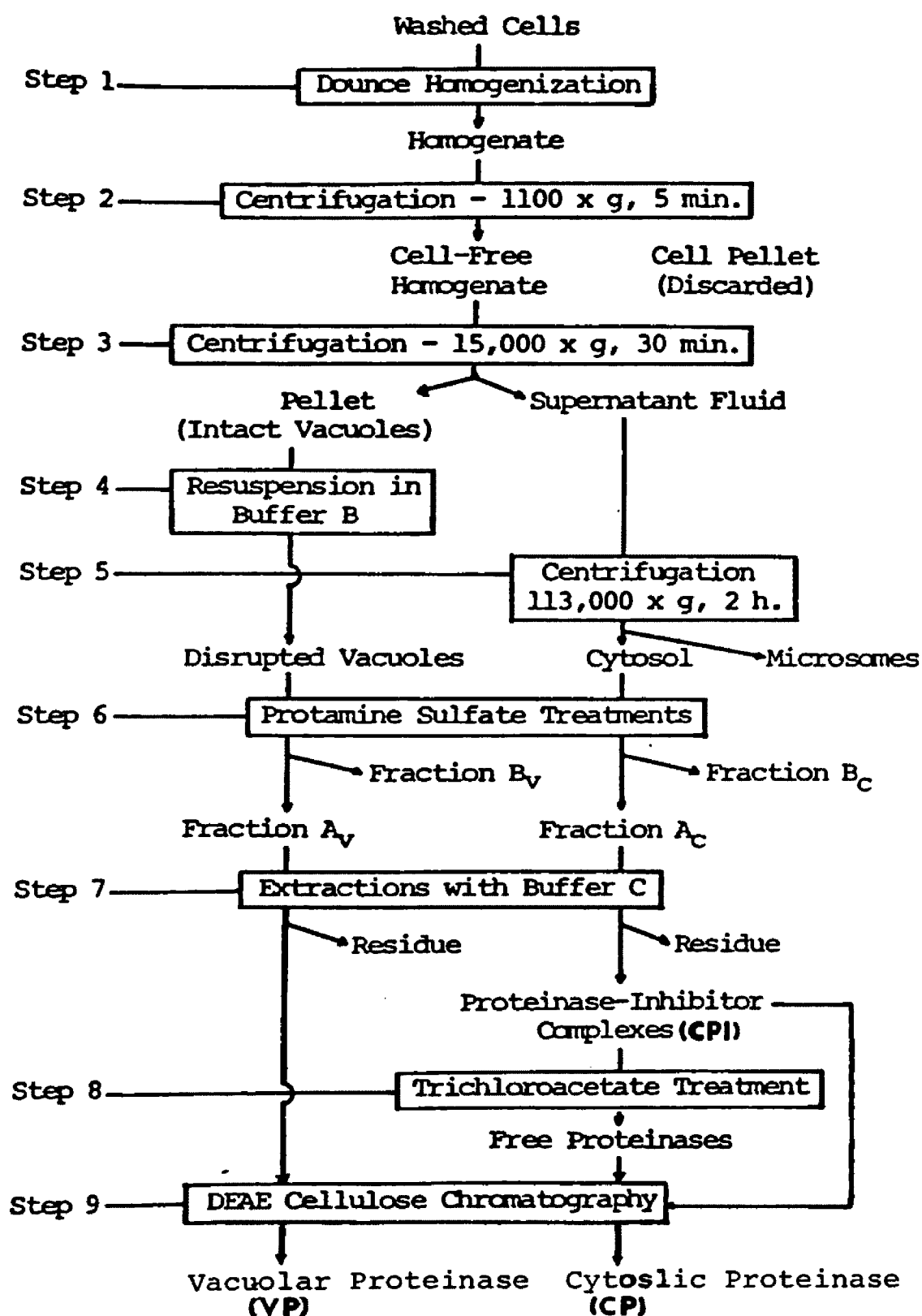
The assay for masked esterase activity was routinely evaluated in the same manner as above, except that the assay buffer was Buffer F. Buffer F, 1.3 M TCA.NaAc, was prepared prior to use by slowly mixing equal volumes of 8 M trichloroacetic acid and 8 M sodium hydroxide, then adjusting the pH to 5.5 with 0.2 M sodium acetate.

3. SUBCELLULAR FRACTIONATION

A. Disruption of cells

In step 1, cells were suspended in Buffer A at a density of approximately 1.5×10^9 cells/ml (Figure 1). The "A" pestle was used with a Dounce homogenizer, and 40 strokes were applied. In step 2, the homogenate was diluted with 2 volumes of Buffer A and the resulting mixture was centrifugated at $1,100 \times g$ for 5 minutes in order to remove all intact cells.

Figure 1. The Outline of Subcellular Fractionation Procedure.



B. Isolation of cytosolic and vacuolar fractions

In step 3 (Figure 1), the homogenized cells were centrifuged at 15,000 xg for 30 minutes in a Sorvall centrifuge equipped with an SS-34 rotor at 4°C. The pellet containing intact vacuoles was recovered and resuspended in Buffer B (step 4). This resuspension resulted in a rapid osmotic lysis of vacuoles. The supernatant fluid contained the cytosol and some membranous material (possibly from the ER). In step 5, membrane-free fractions of vacuole contents and cytosol were prepared by centrifugating the fractions from step 3 & step 4 at 113,000 xg for 2 hours at 4°C in a Beckman L2-65B ultracentrifuge, utilizing SW 28 type rotor.

4. PURIFICATION OF VACUOLAR PROTEINASE

A. Protamine sulfate treatment

In step 6 (Figure 1), the membrane-free vacuolar fraction was mixed with one-tenth volume of an aqueous 5% solution of protamine sulfate. The pH was adjusted to 6.5-6.7 by addition of a small amount of 1 N NaOH. After stirring for 10 minutes, the mixture was resolved into soluble and precipitated subfractions by centrifugation at 3,100 xg for 5 minutes. The supernatant fluid was designated fraction Bv. The pellets were washed by centrifugation in 100 ml of cold, distilled water. Proteinases in the pellet were extracted with Buffer C, and this extract was designated as fraction Av (step 7, Figure 1).

B. DEAE-Cellulose Chromatography

Fraction Av was applied to a column (1.5 cm x 8 cm) of Whatman DE52 cellulose, previously equilibrated with 50 ml of 10 mM Tricine (pH 8.0), 0.5 mM DTT. The column was developed by elution with 50 ml of the above buffer followed by elution with a 200 ml linear NaCl gradient from 0.0 to 0.5 M (in 10 mM Tricine, pH 8.0, containing 0.5 mM dithiothreitol). The flow rate was approximately 0.5 ml / minute, and 5 ml fractions were collected and assayed for esterase activity in Buffer E as described above. The majority of vacuolar proteinase was collected as the major peak of esterase activity and eluting at the salt concentration of approximately 0.30 M NaCl. This fraction was designated as the vacuolar proteinase (VP).

5. PURIFICATION OF MASKED CYTOSOLIC PROTEINASES

A. Protamine sulfate treatment

The cytosolic fraction was fractionated with protamine sulfate by the same method described above. The protamine sulfate non-precipitated fraction was collected and designated as fraction Bc. The protamine sulfate precipitated fraction was washed by centrifugation in the cold distilled water, and then extracted with Buffer C (step 7, Figure 1). This extract was designated as fraction Ac.

B. DEAE-Cellulose chromatography

Cytosolic fraction Ac was subjected to DEAE-cellulose chromatography using the same method described in Section 4.B. The majority of cytosolic proteinase was collected as a major peak of proteinase-inhibitor complex. This complex was assayed by the method described in Section 2.B., and was designated CPI.

C. Sephadex G-200 chromatography

A 1.7 cm x 47 cm column of Sephadex G-200 was equilibrated by elution with 150 ml of 10 mM Tricine (pH 8.0) containing 0.5 mM DTT. One ml of proteinase sample (approximately 10 units total esterase activity) was applied to the column, and the column was developed by elution with 150 ml of the equilibration buffer. The flow rate of the column was 0.12 ml per minute and the temperature was maintained at 4°C. One ml fractions were collected and assayed in Buffer B for masked esterase activity (Section 2.B.).

6. PURIFICATION OF UNMASKED CYTOSOLIC PROTEINASES

A. Trichloroacetate-treatment

One-half volume of 3.2 M TCA.NaAc (pH 5.5) was added to a preparation of cytosolic fraction Ac, and this mixture was adjusted to pH 5.5 by addition of glacial acetic acid. After a 30 minutes incubation period the mixture was diluted with 10 volumes of Buffer B and adjusted to pH 8 by addition of a few

drops of 1 N NaOH - yielding a solution of unmasked proteinase. The solution was dialyzed using membrane tubing with a 3500 MW cutoff to remove the chaotrope, and subsequently concentrated by pressure ultrafiltration in an Amicon concentrator with a YM 5 membrane.

B. DEAE-Cellulose chromatography

The TCA-treated cytosolic proteinase was subjected to a DEAE-cellulose chromatography by the same method described above. The majority of proteinase activities was collected as the major peak of esterase activity assayed in both Buffer E and F, and designated cytosolic proteinase (CP).

C. Sephadex G-200 chromatography

The unmasked cytosolic proteinase (CP) was finally purified by Sephadex G-200 chromatography with the same method described above.

7. DIALYSIS AND SAMPLE CONCENTRATION

Samples requiring exhaustive dialysis were dialyzed using membrane tubing with a 3500 MW cut off. Sample with low concentrations of enzyme were concentrated by pressure ultrafiltration in an Amicon concentrator with a YM 5 membrane.

8. PROTEIN ASSAY

Protein was measured spectrophotometrically (30). Samples were measured at 260 nm and 280 nm. The protein concentration (mg protein/ml) was calculated with the following formula:

$$1.45 (\text{OD}_{280}) - 0.74 (\text{OD}_{260}) = \text{mg protein/ml}$$

9. ELECTROPHORESIS STUDIES

A) Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (32) in 14 x 16 cm vertical slab gels. The gels consisted of two layers - a separating gel and a stacking gel. The separating gel (12.5%) was prepared by mixing together the following solutions in a 125 ml Erlenmeyer flask: 12.5 ml of stock acrylamide (30 g : 0.8 g, acrylamide : bis-acrylamide), 6 ml of 1.875 M Tris buffer (pH 8.8), 0.3 ml of 0.2 M EDTA, 10.9 ml of distilled water, 0.015 ml of TEMED (N,N,N',N'-tetramethylethylenediamine, 99% stock) and 0.3 ml of a 10% ammonium persulfate stock solution. This preparation was mixed thoroughly and approximately 25 ml were poured between the plates of the electrophoresis apparatus. The separating gel was allowed to polymerize for 20 minutes. During this time, the stacking gel was prepared by mixing the following components in a 125 ml flask: 2.5 ml of acrylamide (same solution as described above), 1.88 ml of 1 M Tris buffer (pH 6.8), 0.15 ml of 0.2 M EDTA, 10.3 ml of distilled water, 0.0075 ml of TEMED

and 0.15 ml of a 10% stock solution of ammonium persulfate. This preparation was mixed well and 8 ml were poured into the electrophoresis apparatus on top of the polymerized separating gel. The desired comb was then inserted into the stacking gel solution. Polymerization of stacking gel was complete after 45-50 minutes.

Running buffer (pH approximately 8.3) was prepared by dissolving 3.025 g of Trizma Base, 14.4 g Glycine and 1.0 g of SDS in sufficient distilled water to give a final volume of one liter. The running buffer was distributed equally between the upper and lower reservoirs of the electrophoresis apparatus. Proteinase samples were mixed with equal volumes of 2 X sample solubilizing solution (0.062 M Tris-HCl, pH 6.8, 2% SDS, 10% Glycerol, 4% 2-Mercaptoethanol and 0.001% Bromophenol blue). Samples were solubilized at 100°C for 5 minutes. Electrophoresis was performed at 50 mA until the tracking dye (Bromophenol blue) had reached the bottom of gel (about 4 hours). The gel was removed from the electrophoresis apparatus and stained in Stainsall as described by Green (28).

B. Non-dissociating polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed under non-dissociating conditions (ND-PAGE) essentially as described above. However, 0.1% SDS was deleted from the buffer system. Samples were prepared without 2% SDS and were not subjected to

heat treatment. Electrophoresis was performed at 50 mA for 4 hours. The gel was removed from the electrophoresis apparatus and stained as described above.

10. IMMUNOLOGICAL ANALYSES

A. Electroblotting

Blotting buffer was prepared as a 10 X stock solution (0.2 M Sodium Phosphate, pH 7.4) and was diluted immediately prior to use to a final concentration of 0.02 M phosphate (400 ml of stock blotting buffer was mixed with 3600 ml of distilled water). 2500 ml were poured into a water cooled electroblotting apparatus (dimensions 10 x 20 x 28 cm) and cooled for two hours. The remaining 1500 ml were used to equilibrate the nitrocellulose membrane required for blotting.

Proteins for immunological studies were first resolved on a 15% (or 12.5%) SDS-PAGE gel. The gel was removed from the electrophoresis apparatus and rinsed with distilled water. The gel was layered onto a sheet of nitrocellulose membrane (10 x 15 cm) previously equilibrated in the 0.02 M sodium phosphate buffer. The gel and the nitrocellulose membrane were sandwiched between two pieces of filter paper. This sandwich was placed between two Scotch-Brite pads and inserted into a plastic holder. This entire assembly was then placed into the blotting chamber containing the blotting buffer. Proteins were electrophoretically transferred to the nitrocellulose membrane

(toward the positive electrode) at 24 volts for 15 hours.

B. Reactions of electroblots with specific antisera

The nitrocellulose membrane was removed from the electroblotting chamber and equilibrated for 1 hours in TTBS (20 mM Tris-buffered Saline, pH 7.4, containing 0.05% Tween-20) and then probed with a 1:200 dilution of Anti-Pr1 antiserum; 1:200 dilution of Anti-Pr1-HF antiserum; 1:50 dilution of Anti-N-Acetyl-glucosamine-1-Phosphate-Affinity purified antibody; or 1:50 dilution of Anti-inhibitor antiserum for a 2 hours incubation. All antisera dilutions were in TTBS. After incubation, the membrane was rinsed with three changes of TTBS for 10 minutes each, followed by incubation for 1 hour with a 1:300 dilution of antibody conjugate Goat-Anti-Rabbit-Horse Radish Peroxidase (GAR-HRP). After incubation, the nitrocellulose membrane was again rinsed with three changes of buffer, however, the last 10 minute rinse was in TBS (Tris buffered saline: TTBS without Tween-20). Developer was prepared immediately prior to use by adding 0.66 mg of 4-Chloro-1-Naphthol to 20 ml of cold methanol. This was mixed with 100 ml of TBS containing 60 μ l of H₂O₂ (8.8 M). The naphthol derivative served as a chromogenic substrate for the conjugated Horse Radish Peroxidase. The nitrocellulose membrane was incubated in this solution until optimal development occurred (usually 15-20 minutes). The reaction was stopped by repeated rinsing in distilled water.

11.EFFECT OF TCA.NaAc AND PH ON UNMASKING OF CPI AND VPI

A series of 1.3 M TCA.NaAc buffers covering a pH range of 4.5 to 6.5 were prepared as described previously. To prepare CPI and VPI, CP (5 units/ml) and VP (9 units/ml) were mixed with sufficient inhibitor to totally mask enzyme activity as judged in the standard esterase assay, Buffer E. Masked CPI and VPI (0.010 ml) were incubated individual with the 0.100 ml portions of 1.3 M TCA.NaAc buffers with different pH conditions and at room temperature. At designated times, samples were brought to pH 5.5 by addition of 0.66 M NaAc buffer, pH 5.5 (0.830 ml) and supplemented with 0.010 ml of 100 mM DTT. These mixtures were then supplemented with 50 ml of esterase substrate (5 mM CBZ-Lys-ONp in 50% acetonitrile) and evaluated for esterase activity as described previously. The relative esterase activity of each sample was measured with the control of CP (5 units/ml) or VP (9 units/ml).

RESULTS

1.SUBCELLULAR DISTRIBUTION OF PROTEINASE

Bacteria free stationary phase amoeba of Dictyostelium discoideum were disrupted in a Dounce homogenizer and whole cells removed as described in Methods. The cell free homogenate was then centrifuged at 15,000 xg for 30 minutes. The pellet containing lysosomes, food vacuoles, peroxisomes, and other cellular organelles, was designated as the "vacuolar fraction". The supernatant fluid containing cytoplasmic material and some membranes, perhaps from the endoplasmic reticulum, was further resolved into a cytosolic fraction and a microsomal fraction by high speed centrifugation at 113,000 xg for 2 hours (Figure 1).

As shown in Table 2, about 77% of the total proteinase activity was recovered in the vacuolar fraction in a free, unmasked form. About 12% of the total proteinase activity was recovered in the cytosolic fraction. The cytosolic proteinase activity was detected only when assayed in the presence of 1.3 M sodium trichloroacetate (TCA.NaAc). The unmasking of activity with TCA.NaAc indicated that the cytosolic proteinase was complexed with inhibitor (19).

Table 2. Summary of Subcellular Distribution of Proteinase and Proteinase Purification.

Step	Fraction	Units Proteinase		**Yield %	
		TCA-NaAc	NaAc	v	c
Step 1	Whole Cell	1778	ND		
Step 5	Vacuolar	1222	1484	77	
	Cytosolic	200	15		12
	Microsomal	178	ND		11
Step 6	Av	572	ND	35	
	Bv	193	ND	12	
	Ac	82	6		4.6
	Bc	118	4		6.6
Step 8	Ac*	70	60		3.9
Step 9	Av-DEAE (VP)	558	ND	34	
	Bv-DEAE	126	ND	7.1	
	Ac-DEAE (CPI)	68	0		3.8
	Ac* -DEAE (CP)	55	49		3.1

*: The preparation after TCA-treatment.

**: The yield percentage measurement was using of assay buffer in the presence of 1.3 M TCA at pH 5.5.
(5.8×10^{10} cells total)

2. PURIFICATION OF VACUOLAR PROTEINASE

A. Protamine sulfate treatment

One-tenth volume of an aqueous 5% protamine sulfate solution was added to the vacuolar fraction. Two subfractions of enzyme were obtained. Subfraction Av represented esterase activity that precipitated as a complex with protamine sulfate and was recovered by extraction of this precipitate with 10 mM tricine / 0.3 M NaCl (pH 8). About 75% of the total vacuolar proteinase activity was recovered in the subfraction Av. Subfraction Bv represented esterase activity which did not precipitate with protamine sulfate and was recovered in the supernatant fluid of the protamine sulfate extract. About 25% of the total vacuolar esterase activity was recovered in the subfraction Bv (Table 2). Several observations have indicated that the proteinase in the protamine sulfate precipitated fraction Av is uniquely rich in phosphoglycosyl groups (26).

B. DEAE-cellulose chromatography

The elution patterns of esterase activity which were obtained during DEAE-cellulose chromatography of fraction Av and Bv, are shown in Figure 2. The vacuolar fraction contained only free proteinases and fractions were assayed in standard assay Buffer E (see Methods). The panel B of Figure 2 shows a composite of the separate chromatographies of the subfractions Av and Bv. The major vacuolar proteinase was collected as the major peak of the Av subfraction at a salt concentration of

approximately 0.30 M NaCl, and it was designated as VP1. The subfraction Bv was designated as VP2.

3. PURIFICATION OF MASKED CYTOSOLIC CYSTEINE PROTEINASES (CPI)

A. Protamine sulfate treatment

One-tenth volume of an aqueous 5% protamine sulfate solution was added to the cytosolic fraction. Two subfractions of enzyme were obtained. Subfraction Ac represented the esterase activity which precipitated as a complex with protamine sulfate and was recovered by extraction of this precipitate with 10 mM tricine / 0.30 M NaCl (pH 8). Proteinase activity recovered in the subfraction Ac represented about 41% of total cytosolic proteinase (Table 2). Subfraction Bc represented esterase activity which did not precipitate with protamine sulfate and was recovered in the supernatant fluid of the protamine sulfate treated extract (Figure 1).

B. DEAE-cellulose chromatography

The elution patterns of esterase activity which were obtained during DEAE-cellulose chromatography of subfraction Ac and Bc, are shown in panel A, Figure 2. The cytosolic fractions contained the majority of masked proteinase-inhibitor complexes. The proteinase activity was only detected by assaying chromatography fractions with Buffer F in the presence of 1.3 M TCA.NaAc (panel A, Figure 3). The major cytosolic proteinase was collected as the major peak of the

subfraction Ac at a salt concentration of approximately 0.27 M NaCl and it was designated as CPI.

C. Sephadex G-200 chromatography of CPI

The elution pattern of CPI that was obtained during chromatography with Sephadex G-200 is shown in upper panel of Figure 4. During Sephadex G-200 chromatography the enzyme activity eluted in one major peak with a relative elution volume of approximately 1.2. The proteinase activity was detected by assaying chromatography fractions with Buffer F.

4. PURIFICATION OF UNMASKED CYTOSOLIC PROTEINASE

A. Trichloroacetate treatment

The 1.3 M TCA.NaAc buffer (Buffer F, pH 5.5) was routinely used to assay the masked proteinase-inhibitor complexes involving *D. discoideum* cysteine proteinase. It was demonstrated previously that this high concentration of the chaotrope, sodium trichloroacetate, provided for a dissociation of proteinase-inhibitor (CPI) complexes (19).

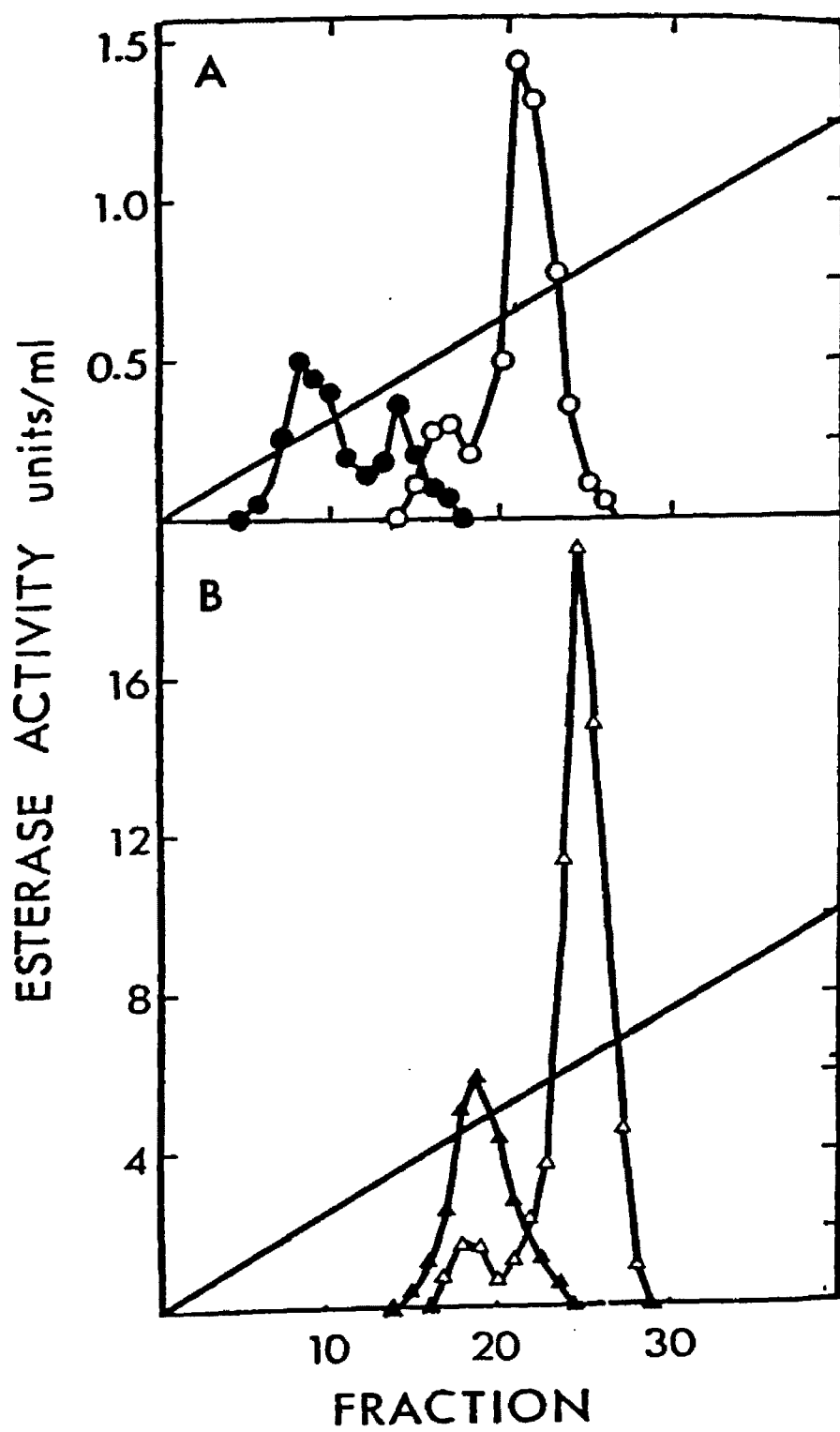
To perform this TCA.NaAc treatment, one-half volume of 3.2 M TCA.NaAc (pH 5.5) was added to the preparation of CPI and subsequently dialyzed to remove the chaotrope (see Methods for details). The results of step 8 in Table 2 show that the unmasked cytosolic proteinase activity could be detected with either Buffer E or Buffer F. This observation supported the

Figure 2. Assays of Proteinases resolved from Subcellular Fractionation.

Cytosolic and vacuolar fractions were prepared by Dounce homogenization and centrifugation of freshly harvested D. discoideum amoeba (5×10^{10} cells total). Samples of each fractions were chromatographed on DEAE-cellulose columns (1.5 x 12 cm). The columns were developed with a 400 ml linear gradient of NaCl containing 10 mM Tricine-NaCl (pH 8) and 0.5 mM DTT (see Materials and Methods in detail).

The upper panel shows a composite of the separate chromatographies of subfractions Ac (o - o) and Bc (●-●) derived from the cytosol. The chromatography fractions (5 ml) were evaluated for esterase activity in reaction mixtures containing 1.3 M Sodium Trichloroacetate (TCA.NaAc, pH 5.5).

The lower panel shows a composite of the separate chromatographies of subfractions Av (Δ - Δ) and Bv (\blacktriangle - \blacktriangle) derived from the vacuolar fraction. These chromatography fractions contained only free proteinases and were assayed in the 0.2 M NaAc (pH 5.5).



view that cytosolic proteinase degraded the inhibitor or the inhibitor was denatured in the presence of high concentrations of TCA-NaAc. The TCA-NaAc-treated, unmasked cytosolic proteinase, was collected and designated as free cytosolic proteinase (CP).

B. DEAE-cellulose chromatography of CP

The free cytosolic proteinases were subjected to DEAE-cellulose chromatography. The lower panel of Figure 3 shows the elution pattern obtained in this chromatography. The peak of proteinase activity eluted at a salt concentration of approximately 0.27 M NaCl was collected and designated as CP. Its chromatographic property was identical to that of CPI (upper panel, Figure 3). CP could be assayed in both Buffer E and Buffer F (lower panel, Figure 3).

C. Sephadex G-200 chromatography of CP

The cytosolic proteinase (CP) was further purified by Sephadex G-200 chromatography. The proteinase activity eluted in one major peak with a relative elution volume of approximately 1.6 (Figure 4).

Figure 3. DEAE-Cellulose Chromatography of CP and CPI.

Assays of esterase activity resolved from the cytosolic fraction of D. discoideum amoebae (5×10^{10} cells total).

The upper panel shows the results of masked cytosolic proteinases (CPI) resolved from DEAE-cellulose chromatography. The proteinase activities were only detected in the 1,3 M TCA.NaAc buffer (o - o), while it had no detectable proteinase activity in the 0.2 M NaAc assay buffer (●-●).

The lower panel shows the results of unmasked cytosolic proteinases (CP) resolved from DEAE-cellulose chromatography. The cytosolic proteinase activities could be detected in the both 1.3 M TCA.NaAc buffer (o - o) and 0.2 M NaAc buffer (●-●).

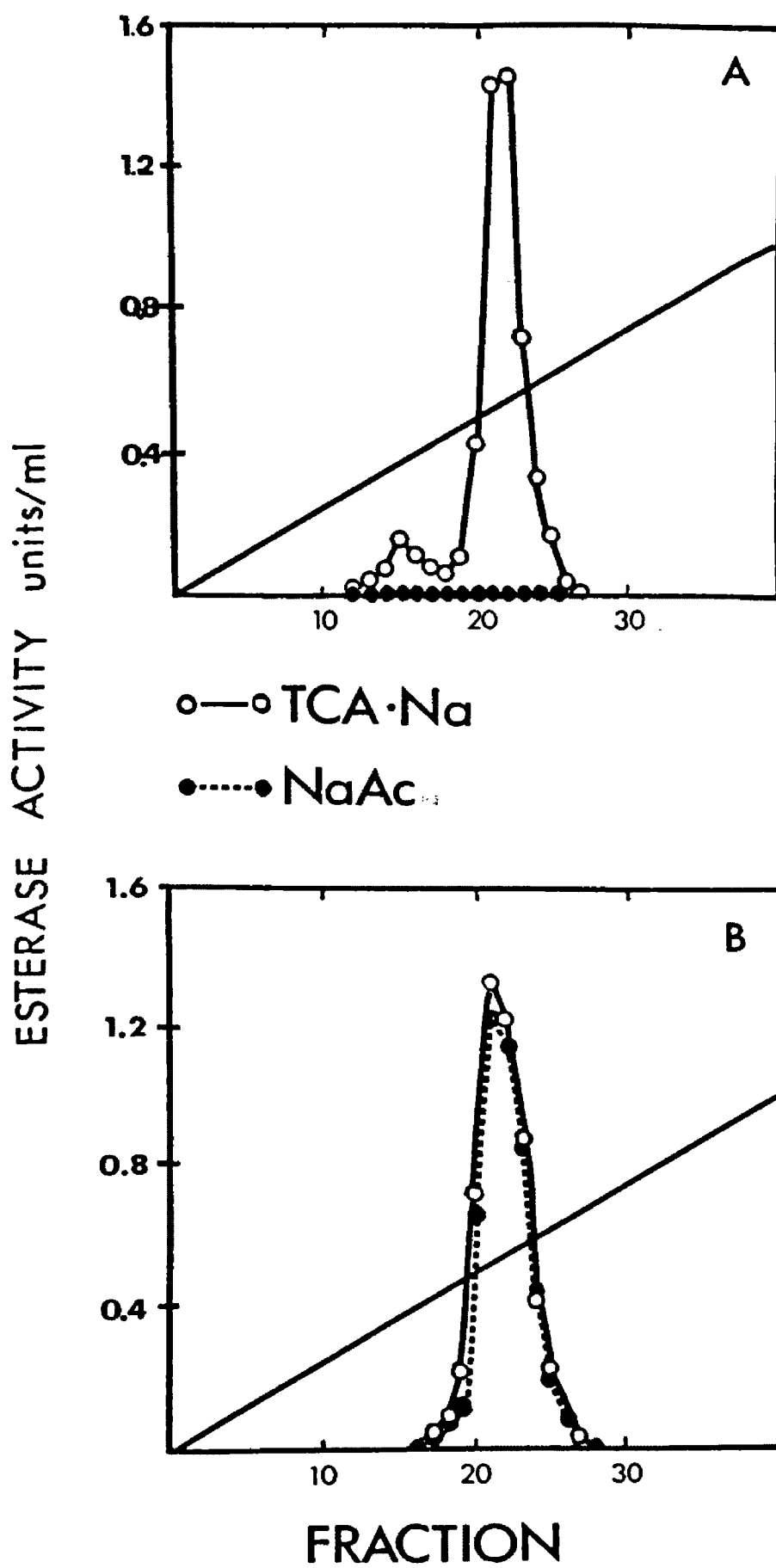
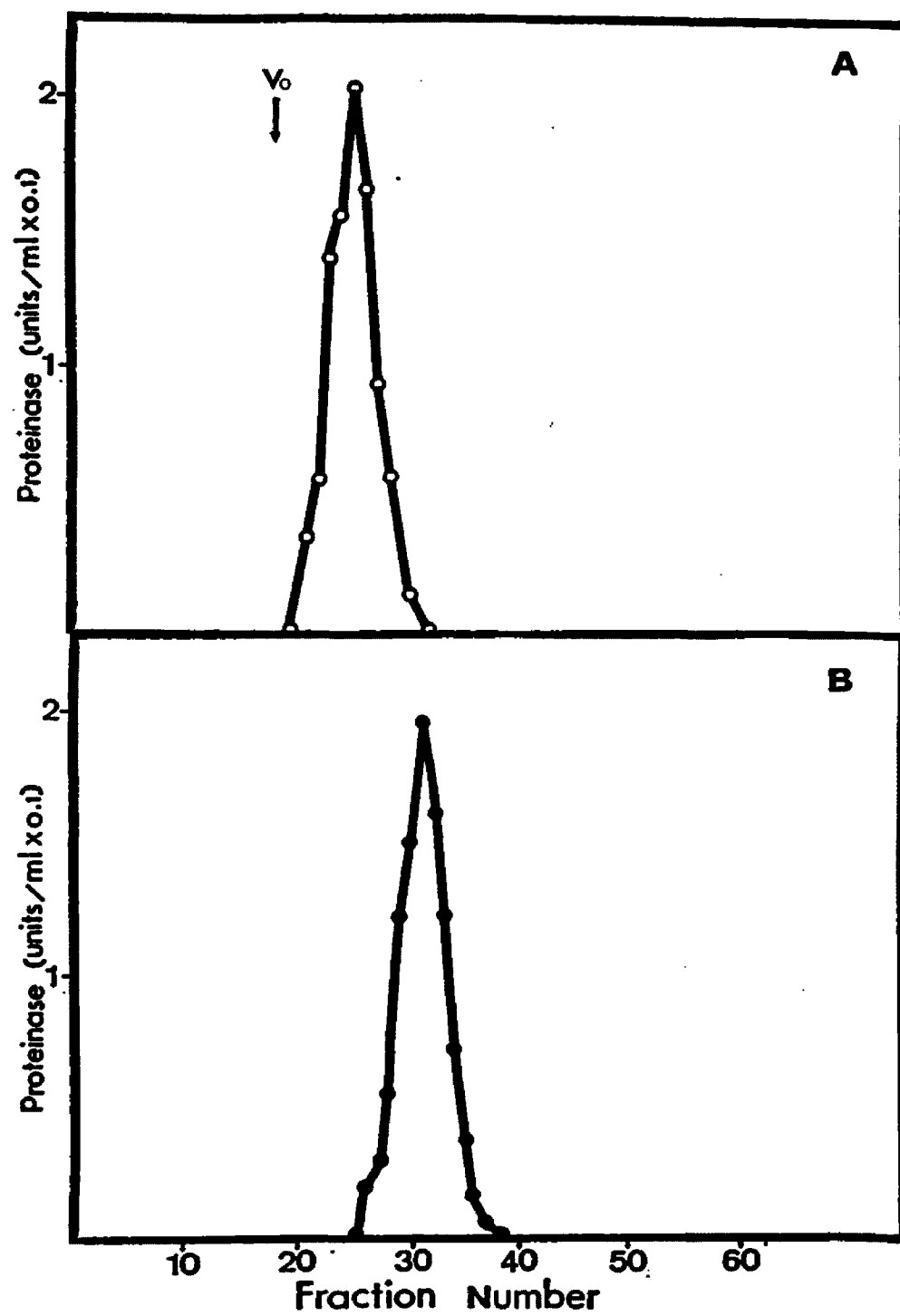


Figure 4. Sephadex G-200 Chromatography of CP and CPI.

Enzyme activity of CP and CPI recovered from DEAE-cellulose chromatography was concentrated by pressure dialysis in an Amicon ultrafiltration cell equipped with a UM-10 filter to a final volume of 1 ml. The resulting solution was then applied to a column of sephadex G-200 previously equilibrated with 10 mM tricine (pH 8.0), 0.5 mM DTT. The column was developed with the same buffer, and 2 ml fractions were collected. The CPI fraction, panel A, were assayed for esterase activities in the 1.3 M TCA.NaAc buffer. The CP, panel B, were assayed for esterase activities in the 0.2 M NaAc buffer. In a separate experiment 1 ml of blue dextran 2000 was chromatographed on the column. The void volume of the column, V_0 , represents the eluted fraction which contained the highest concentration of this polymer.



5. THE PROPERTIES OF VACUOLAR AND CYTOSOLIC PROTEINASES AND THEIR INHIBITOR COMPLEXES

A. ND-PAGE studies

Previous investigations (26), had shown that purified Proteinase 1 (Pr1) preparations, purified from the lyophilized stationary-phase amoebae of D. discoideum, contained a mixture of two protein species. When these two species were resolved by ND-PAGE and stained with Stainsall, both species stained blue (Lane 1, Figure 5). Stainsall is a cationic carbocyanine dye which stains nonphosphorylated proteins red, and phosphorylated proteins blue (28).

For the experiments shown in Figure 5, purified vacuolar proteinase, VP, yielded one blue band. It corresponded to the upper band observed with original preparation of Pr1. A lower band was obtained when free inhibitor was mixed with the preparation of free vacuolar proteinase. This band corresponded to the lower band of Proteinase 1. Apparently, the lower band was derived from the upper band by adding free inhibitor, since the upper band disappeared when a sufficient amount of free inhibitor was added. This suggested that lower band was a proteinase-inhibitor complex. This results also indicated that the Proteinase 1 preparation purified by Gustafson and Thon (16) was a mixture of vacuolar proteinase and vacuolar proteinase-inhibitor complex, and purified VP was an inhibitor-free proteinase.

Figure 5. Non-Dissociating PAGE of Comparison VP and the Mixture of VP with various amount of Inhibitor.

The sample (5 μ l) in lane 1 was the preparation of purified Pr1 (1 mg/ml, 4 units/ml) by Gustafson et al. The sample in lane 2 was the vacuolar proteinase (fraction Av) (20 μ l, 1 unit/ml). Lane 3, 5 μ l of proteinase inhibitor (1 unit/ml) mixed with 20 μ l of fraction Av. Lane 4, 10 μ l of proteinase inhibitor + 20 μ l of fraction Av. Lane 5, 15 μ l of proteinase inhibitor + 20 μ l of fraction Av. Lane 6, 20 μ l of proteinase inhibitor + 20 μ l of fraction Av. The sample preparation of vacuolar proteinase activity in lane 6 was totally unmasked by proteinase inhibitor. The 15% non-denaturing PAGE gel was stained in Stainsall.

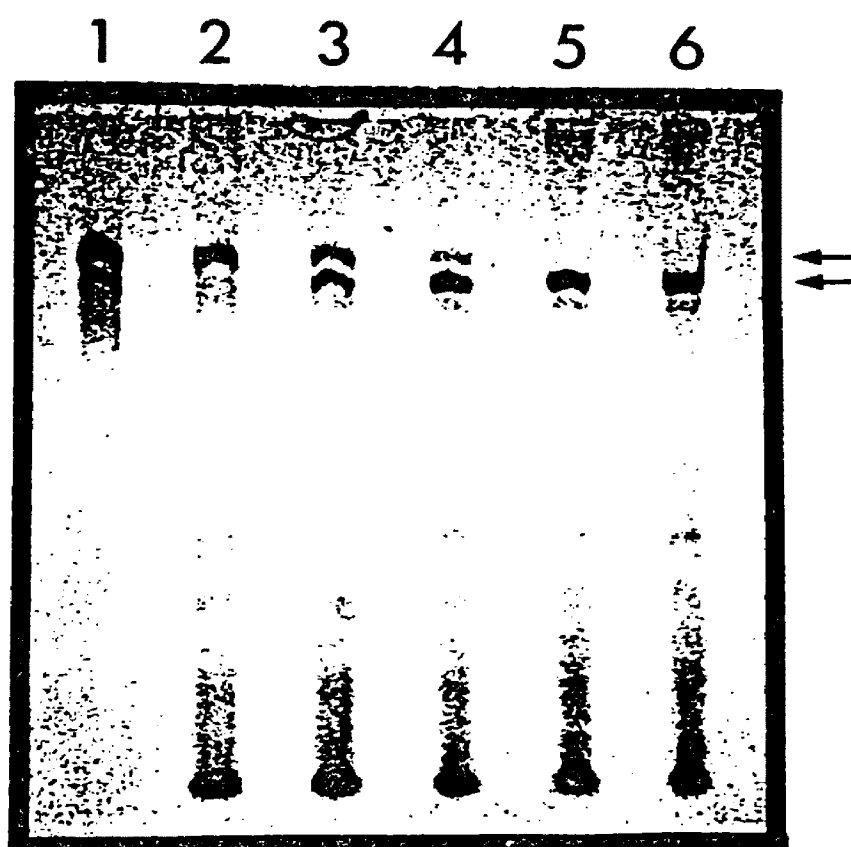
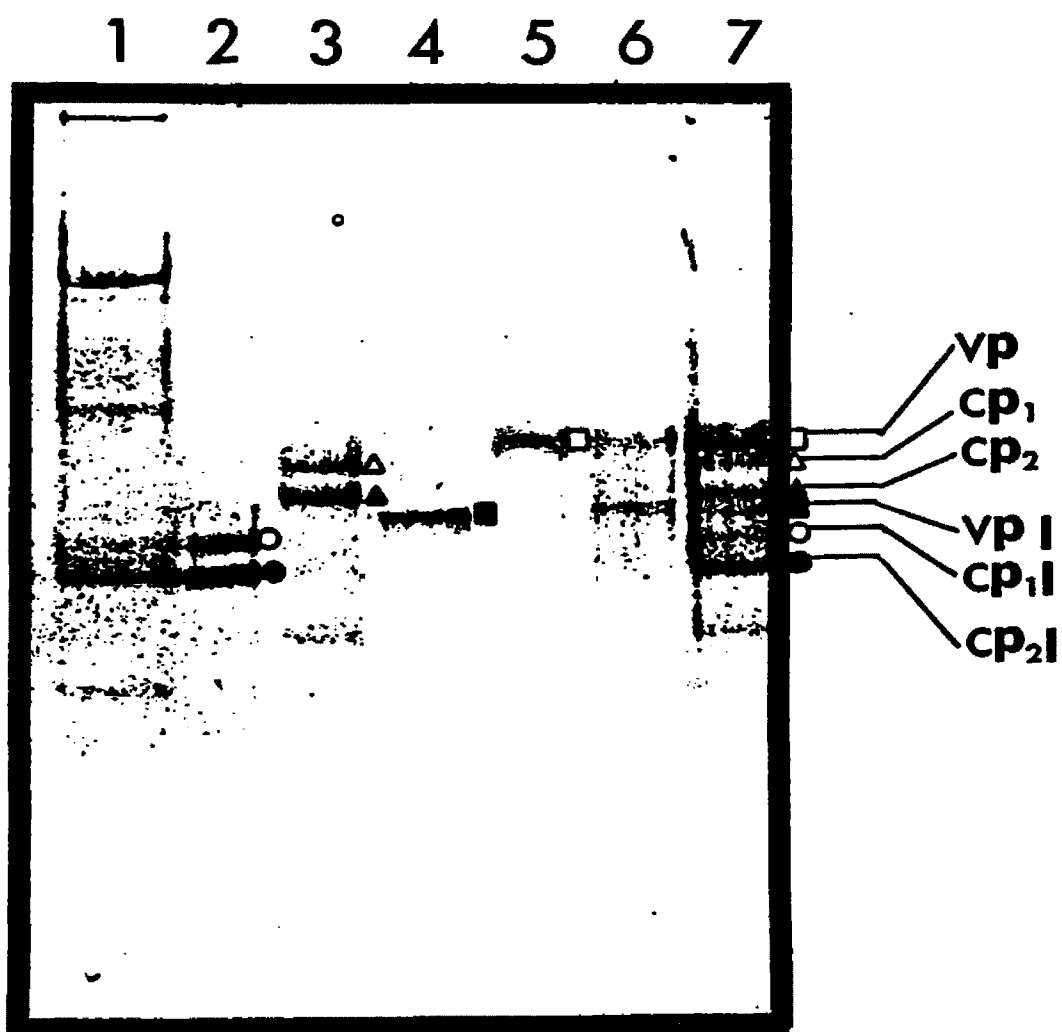


Figure 6 also shows the results of an ND-PAGE study where Stainsall was used. All bands designated by symbols in Figure 6 stained blue, while most other protein bands observed in this figure stained red. The crude cytosolic proteinase (fraction Ac) when collected in masked form yielded two blue bands (lane 1). Two blue bands (with slower mobilities), designated as CP1 and CP2, were observed with the unmasked cytosolic proteinase (lane 3). The results in lane 2 showed that when the unmasked cytosolic proteinases were mixed with excess inhibitor, the free enzyme species were converted into proteinase-inhibitor complexes resembling those observed in lane 1. As with the cytosolic proteinase, vacuolar proteinase (lane 5) exhibited a lesser electrophoretic mobility when it was in an unmasked state (lane 5) than when it was complexed with inhibitor (lane 4). The results shown in lane 7 documented that the two cytosolic proteinases (CP1 and CP2), the vacuolar proteinase (VP), and each of the three respective proteinase-inhibitor complexes could be resolved from one another by ND-PAGE.

Figure 6. Non-Dissociating PAGE of Vacuolar Proteinase, Cytosolic Proteinases and their Proteinase-Inhibitor Complexes.

Samples of proteinases and their proteinase inhibitor complexes were subjected to electrophoresis in a 10% non-denaturing PAGE gel as described in the Methods. The gel was removed from the electrophoresis apparatus and stained in Stainsall. All bands designated by arrows in the figure stained blue, while most other protein bands observed in this figure stained red.

The sample preparation in lane 1 was the crude cytosolic proteinase, fraction Ac (20 μ g). Lane 3 was the preparation of (10 μ g) unmasked cytosolic proteinases (CPs). Lane 2 was the preparation sample of CPs (10 μ g) mixed with sufficient amount of free purified inhibitor. Lane 5 was the preparation of unmasked VP (5 μ g). Lane 4 was the preparation of VPI (5 μ g of VP with sufficient amount of inhibitor). Lane 6 was 5 μ g of Pr1 preparation (5 μ g) purified by Gustafson et al. Lane 7 was the preparation of a mixture of VP, VPI, CP₁, CP₂, CP₁I and CP₂I.

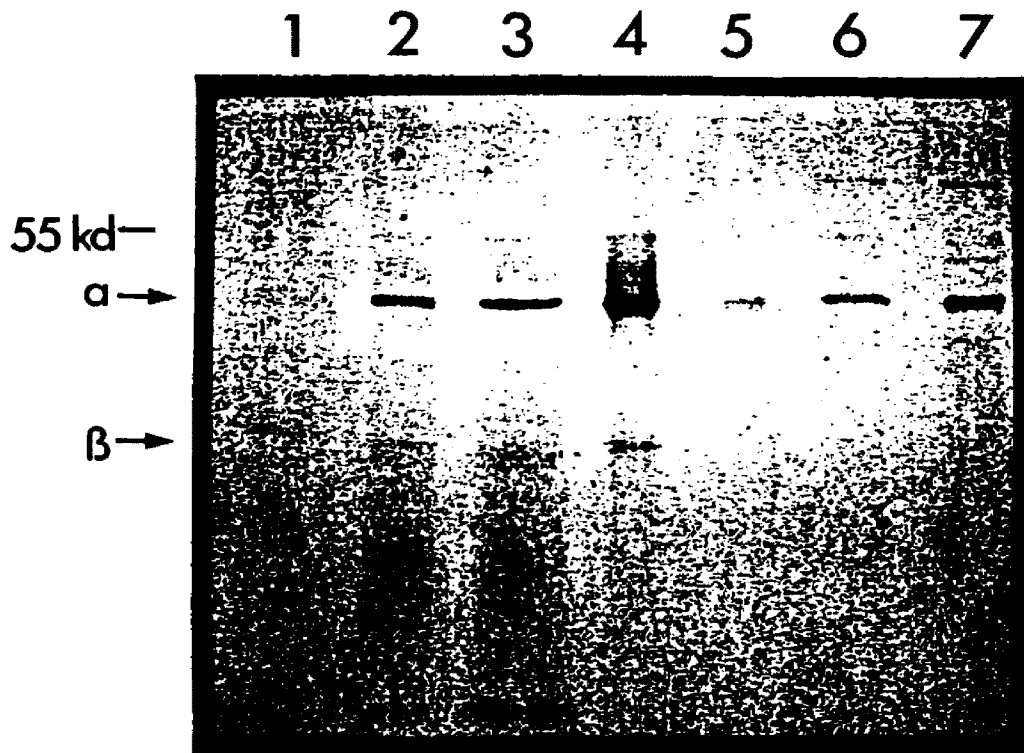


B. SDS-PAGE gel electrophoresis Studies

The results in Figure 7 showed that SDS-PAGE gel electrophoresis resolved the vacuolar proteinase into 2 subunits designated as α and β . The electrophoretic mobilities of these 2 subunits were compared with that of standard molecular weight marker proteins, bovine serum albumin, 66.2 kd; ovalbumin, 45 kd; carbonic anhydrase, 31 kd; soybean trypsin inhibitor, 21.5 kd; and β -lactalbumin, 14.2 kd. The larger subunit α had an apparent molecular weight of about 39 kd, and stained blue with Stainsall. It was common to VP (Lane 2 and 3) and CP (Lane 5, 6 and 7). The smaller subunit β had an apparent molecular weight of about 18 kd, and stained red. It was only found in VP (Lane 2 and 3). A high MW band (about 55 kd) was also observed in VP (Lane 2 and 3) and Pr1 (Lane 4). It also stained blue. Other bands not referred to above that were observed in Figure 7 were assumed to be extraneous proteins.

Figure 7. Comparison of Subunits of Vacuolar and Cytosolic Proteinases in SDS-PAGE.

The sample preparation in lane 1 was 20 μ g of crude vacuolar proteinase, fraction Bv. The sample preparations in lane 2 and 3 were 10 μ g and 20 μ g of crude vacuolar proteinase, fraction Av. The sample preparation in lane 4 was the Pr1 preparation (5 μ g) purified by Gustafson et al. Lane 5, 6, and 7 contained 10 μ g, 20 μ g and 40 μ g of crude cytosolic proteinase (fraction Ac), respectively. The 15% SDS-PAGE gel was used. The band designated by upper arrow in the figure stained blue with Stainsall staining. The lower band and other bands are stained red.



Earlier investigations (26) had suggested that Pr1 was a polymeric enzyme containing 3 subunits, with MW's approximately 55 kd, 39 kd and 18 kd. The results in Figure 8 and 10A, however, suggested that 55 kd band was only formed in preparations of VP and VPI that had been boiled. The analogous results from immunoblot studies (Figure 10A and 10B) also supported this view.

Another interesting result was observed in Figure 8. In comparing the boiled and unboiled proteinase samples prepared in SDS-PAGE sample solubilizing (SSS) buffer, it was noted that unboiled Pr1 preparations resolved as two major blue bands; while unboiled VP preparations resolved as a single blue band. When Pr1 preparation were boiled, the high MW band was lost. However, the 55 kd band was formed in both boiled VP and boiled Pr1 preparation. The results in Figure 9 suggested that high MW band might be vacuolar proteinase-inhibitor complex. As described below the results of experiments presented in Figure 10 (Lane 2, Panel B & C) demonstrated that the high MW band from Pr1 was vacuolar proteinase-inhibitor complex that did not dissociate in the presence of SDS when samples were not boiled. This band was lost upon boiling of samples because of the dissociation of the proteinase-inhibitor complex.

Figure 8. Comparison of Subunits of Vacuolar Proteinase (VP) and Proteinase 1 (Pr1) in SDS-PAGE.

The sample preparations from lane 1 to lane 8 were 5 μ g of Pr1. The other sample preparation from lane 9 to lane 15 were 5 μ g of crude vacuolar proteinase, fraction Av. Lane 1 and 2, Pr1 were incubated with SDS-PAGE sample solubilizing (SSS) solution at room temperature for 0 and 10 minutes before use; lane 3, 30 minutes. Lane 4 and 5, Pr1 were incubated in SSS solution (without SDS) for 10 and 30 minutes and then boiled for 5 minutes. Lane 6, 7 and 8, Pr1 were incubated in SSS solution for 0, 10 and 30 minutes and then boiled for 5 minutes. Lane 9 and 10, VP were incubated with SSS solution at RT for 0 and 10 minutes. Lane 11 and 12, VP were incubated in SSS solution (without SDS) at RT for 10 and 30 minutes and then boiled for 5 minute. Lane 13, 14 and 15, VP were incubated in SSS solution at RT for 0, 10 and 30 minutes before boiled. The 12.5% SDS-PAGE gel was stained in Stainsall.

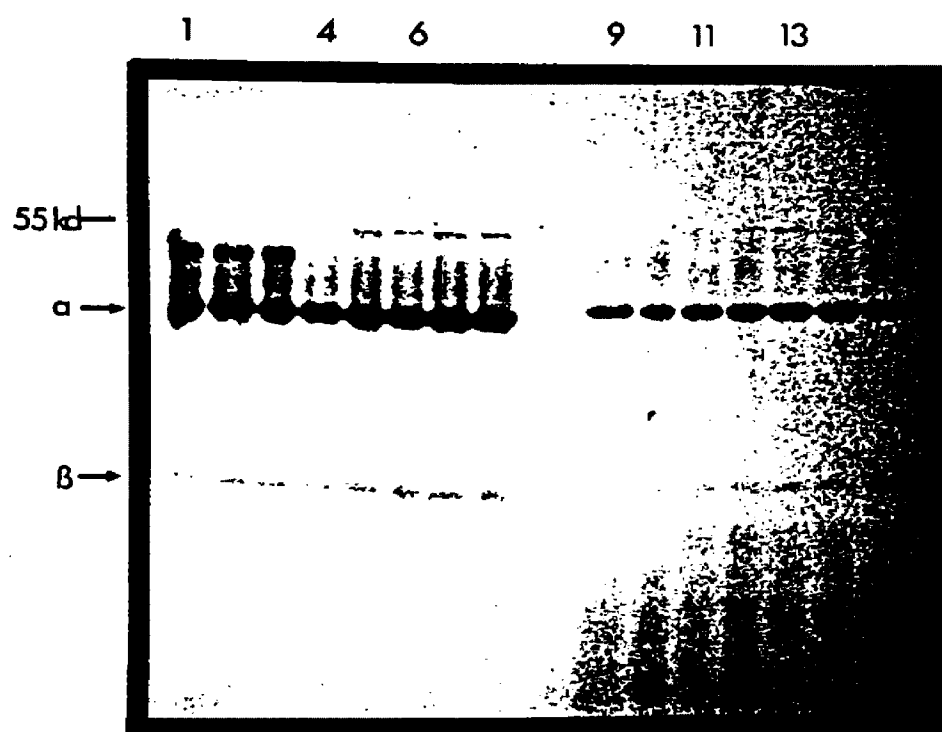


Figure 9. Comparison of Subunits Compositions by adding Inhibitors.

The sample preparation in lane 1 was 20 μ l (1 mg/ml) of crude vacuolar proteinase, fraction Av. Lane 2, 5 μ g of proteinase inhibitor (1 unit/mg) + 20 μ l of fraction Av. Lane 3, 10 μ l of proteinase inhibitor + 20 μ l of fraction Av. Lane 4, 15 μ l of proteinase inhibitor + 20 μ l of fraction Av. Lane 5, 20 μ l of proteinase inhibitor + 20 μ l of fraction Av. The sample preparation in lane 6 was 5 μ l (4 mg/ml) of Pr1. All samples were mixed with SDS-PAGE Sample Solubilizing Solution without boiled and then subjected to the 12.5% SDS-PAGE. The gel was stained in Stainsall.



C. Immunoblot Studies

In Figure 10A, the unboiled VPI sample in lane 1 was incubated in SSS buffer at room temperature for 30 minutes, and VPI sample in lane 2 was incubated in a boiling water bath with SSS buffer for 5 minutes. Both samples were subjected to SDS-PAGE gel electrophoresis and the resulting gel was stained with Stainsall. Each high MW band (●) and α band of unboiled VPI (Lane 1), and the 55 kd band (■) and α band of boiled VPI (Lane 2) were excised from the gel. These gel slices were boiled individually in SSS buffer for 5 minutes and then subjected to a second SDS-PAGE. The second SDS-PAGE gel was analyzed by immunoblot analyses using anti-Pr1-HF and anti-I antiserum.

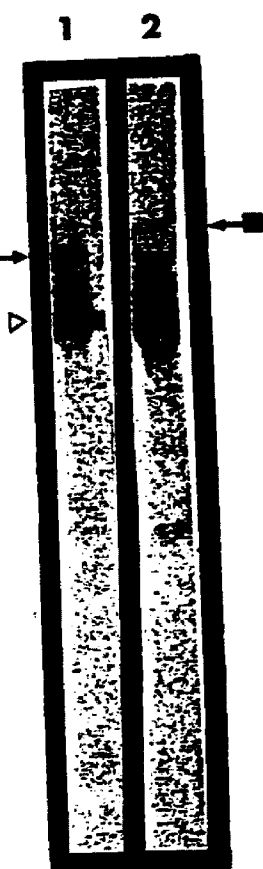
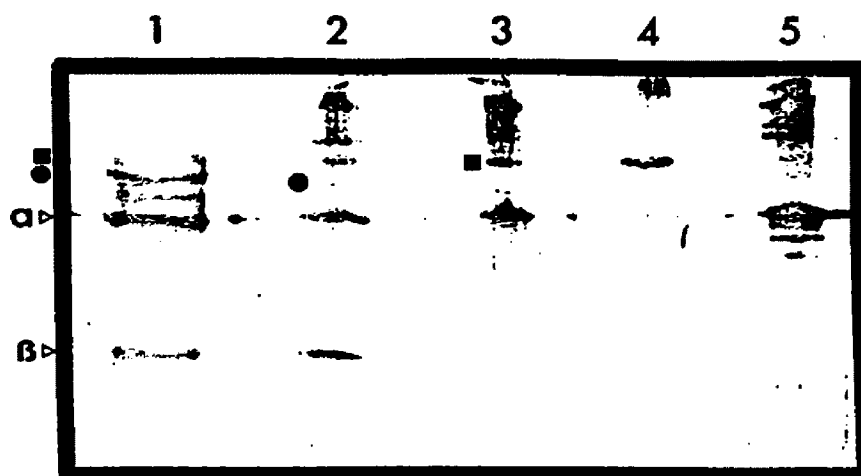
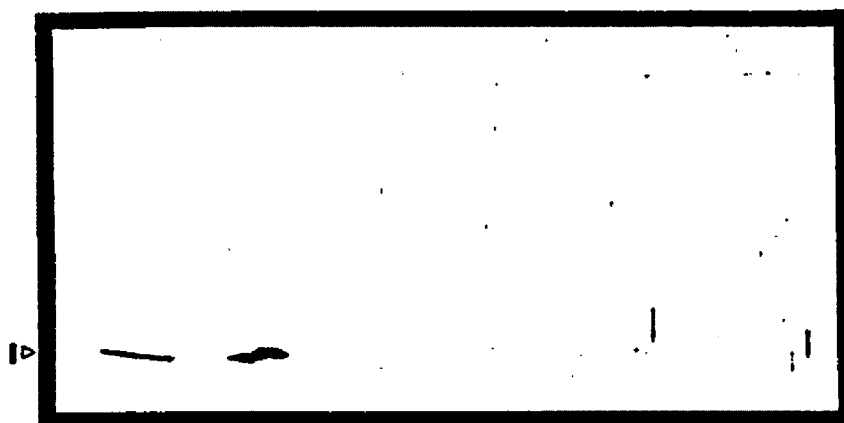
The results in Figure 10B and 10C suggested that high MW band (●) was a proteinase-inhibitor complex that did not dissociate in the presence of SDS unless samples were boiled (Lane 2, Figure B & C). The sample in Lane 3 was resolved from α subunit band of unboiled VPI in the first SDS-PAGE gel. A 55 kd band formed in Lane 3. This suggested that the 55 kd band might be formed by boiling - perhaps representing a dimer of subunits. When the 55 kd band was sectioned from a first SDS-PAGE gel and subjected to a second SDS-PAGE, it again migrated as a single band of 55 kd (Lane 4, Figure 10B). This result indicated that the 55 kd band was non-dissociable after it was formed. The sample in Lane 5 was resolved from the α subunit of

Figure 10. Comparison of high Molecular Weight Subunits formed in the boiled and unboiled VP.

The sample preparations were the equal amount of VPI samples mixed with SSS buffer. The boiled (100°C, 5 min) and unboiled (RT, 30 min) sample preparations of VPI (25 µg of VP + 25 µg of I) were subjected to a first SDS-PAGE gel electrophoresis. Gel was stained with Stainsall (panel A). In Panel A; lane 1, unboiled VPI; lane 2, boiled VPI.

The high MW band (●) and α subunit of unboiled VPI, and the high MW band (■) and α subunit of boiled VPI were excised from the first SDS-PAGE gel (Panel A), boiled within SSS buffer for 5 minutes and then subjected to a second SDS-PAGE gel electrophoresis (12.5% gel). The second was analyzed by Westernblot analysis using 1/200 dilution of anti-Pr1-HF serum (Panel B) or 1/50 dilution of anti-I (Panel C) as probes.

In Panel B and C; Lane 1, unboiled VPI (10 µg of VP + 10 µg of I); Lane 2, the upper band (●) of unboiled VPI; Lane 3, the α band of unboiled VPI; Lane 4, the upper band (■) of boiled VPI; Lane 5, the α band of boiled VPI.

**A****B****C**

the first SDS-PAGE gel. There was no 55 kd band observed in the second SDS-PAGE gel. It was suggested that was only one single α subunit species formed in the α band of boiled VPI.

The experiment employing immunoblots in Figure 11 and 12 were performed to compare the polypeptide compositions of cytosolic and vacuolar proteinases. In Figure 11, individual protein bands resolved by ND-PAGE were excised from gels and subjected at SDS-PAGE. The resulting gel was analyzed by immunoblot analyses using anti-Pr1 antiserum as a probe. In agreement with the results in Figure 7, a common subunit α was detected in all VP, CP1 and CP2. However, different small molecular weight subunits were detected in each proteinase species (ie; β for VP, β' for CP1 and β'' for CP2).

Much clearer immunoblot patterns were observed when proteinase samples were resolved directly by SDS-PAGE electrophoresis (Figure 12). Panel A shows results of immunoblot analyses performed with anti-Pr1 antiserum, the antibodies against the purified Pr1 which consisted of proteinase and inhibitor, as a probe. The common subunit α and different small subunit and β' and inhibitor were detected. The other small subunit β'' was present in too small an amount to detect in this blot. Panel B shows that immunoblot analyses performed with anti-I antibodies raised against purified inhibitor (20), and that the purified preparation of unmasked

vacuolar and cytosolic proteinases contained no inhibitor. Panel C shows results obtained when immunoblot analyses performed with both anti-I and anti-N-acetyl-glucosamine-1-phosphate affinity purified antiserum (27) as probes. Two antigenic bands were detected in a blot of VP, and one band was detect in the blot of CP. The lack of reaction of β' and β'' with affinity antibody suggested these subunits lacked sugar phosphate residues.

Figure 11. Comparison of the Polypeptide Compositions of Cytosolic and Vacuolar Proteinases.

The polymeric forms of VP and CPI samples (25 μ g each) that had been resolved by non-denaturing electrophoresis were excised from gels and subjected to SDS-PAGE (12.5% gel). The resulting gel was analyzed by Westernblot analysis using 1/200 dilution of anti-Pr1 serum as a probe. Lane 1, VP. Lane 2, corresponded to the upper band of CP (Lane 2, Figure 4). Lane 3, corresponded to the lower band of CP (Lane 2, Figure 4).

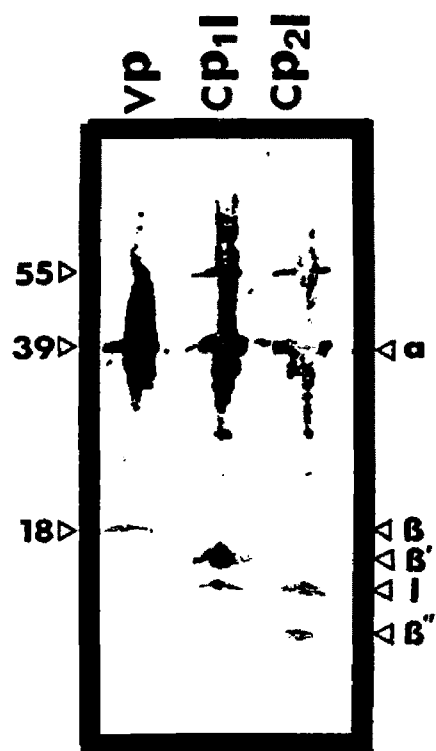
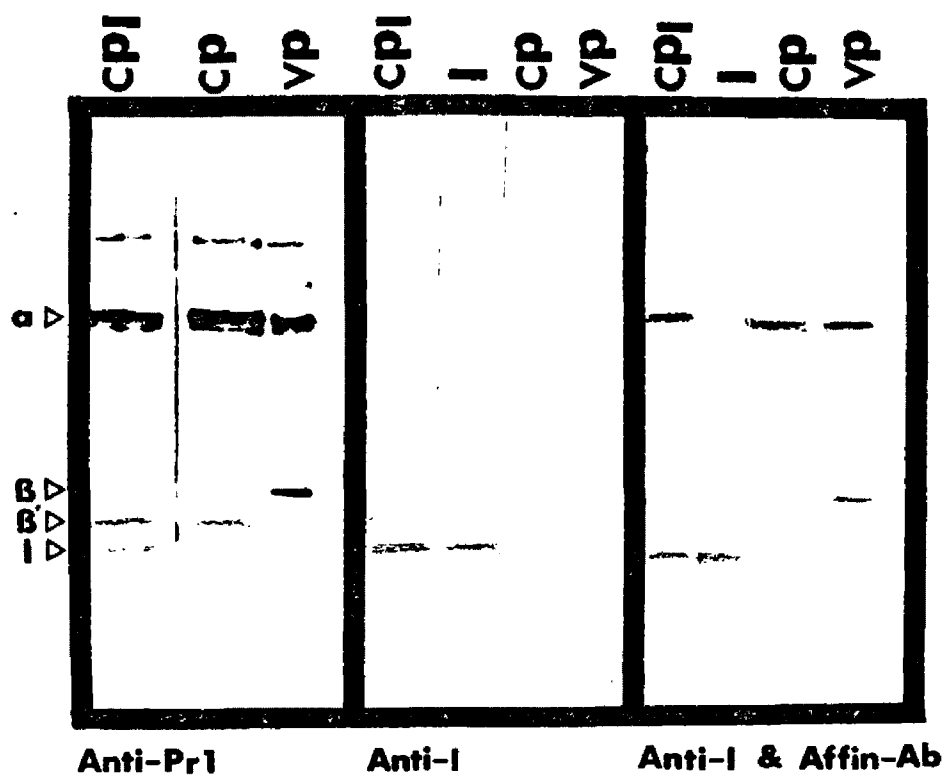


Figure 12. Immunoblot Analysis.

All panels represent blots of proteinases and inhibitor samples (5 μ g of each sample) resolved by SDS-PAGE (12.5% gel) and electrophoretically transferred onto a nitrocellular membrane. Blots in panel 1 were probed with 1/200 dilution of anti-Pr1 serum. Blots in panel 2 were probe with 1/50 dilution of anti-inhibitor serum. Blots in panel 3 were probed with a 1:1 mixture of 1/50 dilution of anti-inhibitor serum and affinity purified anti-N-acetyl glucosamine phosphate antiserum (1 mg/ml IgG).

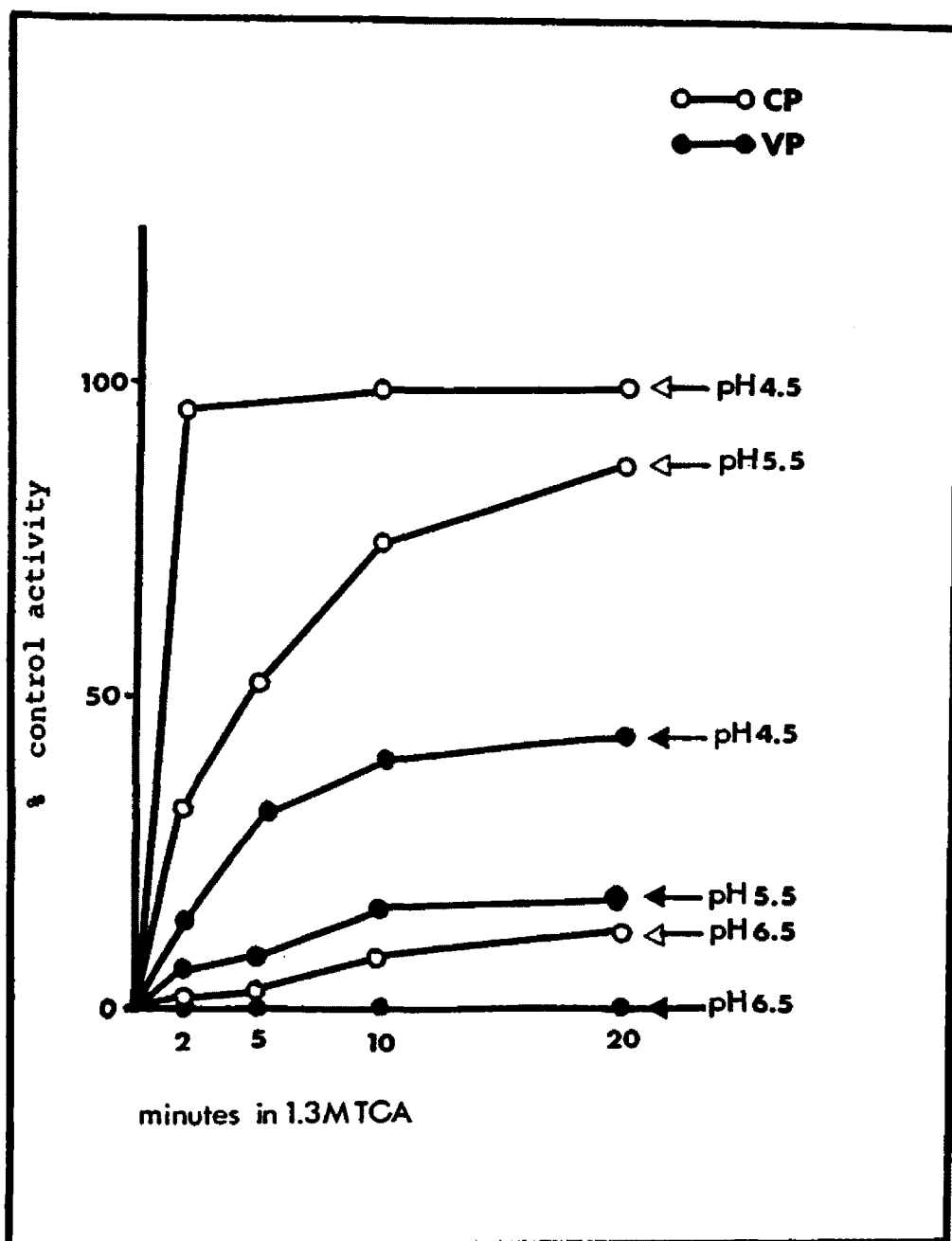


D. Effect of TCA and pH on unmasking of CPI and VPI

The results in Figure 13 show the time dependent unmasking of CPI in solutions of 1.3 M TCA.NaAc at different pHs. The unmasking of proteinase activity was evaluated in assay buffer with a low concentration of TCA. At pH 4.5, exposure of masked cytosolic proteinase to TCA.NaAc for 2 minutes caused a 98% unmasking of esterase activity. In comparison only about 40% of VPI was unmasked in 20 minutes under these conditions. At pH 5.5, exposure of masked cytosolic proteinase for 20 minutes caused a 90% unmasking of esterase activity, but only 15% unmasking of VPI was observed at the same condition. At pH 6.5, 10% unmasking of cytosolic proteinase activity was detected within 20 minutes. However, with this condition no detectable unmasking of vacuolar esterase activity was observed over a 20 minutes period. This observation supported the view that inhibitor might be destroyed by cytosolic proteinases in the presence of high concentration chaotrope, trichloroacetate, at pH 5.5. According to this observation, a TCA-treatment for unmasking of cytosolic proteinases was developed for the purification of free cytosolic proteinases.

Figure 13. Effect of TCA and pH on Unmasking of CPI and VPI.

A constant amount of the VPI (5 μ g of VP + appropriate amount of free inhibitor) and CPI (5 μ g of CP + appropriate amount of free inhibitor) mixtures were incubated in citrate buffers containing 1.3 M TCA.NaAC of designated pH. Samples were evaluated by adding standard esterase assay, Buffer E, to a final volume of 1 ml at designated times. Values were referenced to a 100% activity control of 5 μ g VP and 5 μ g CP assayed in the Buffer E.

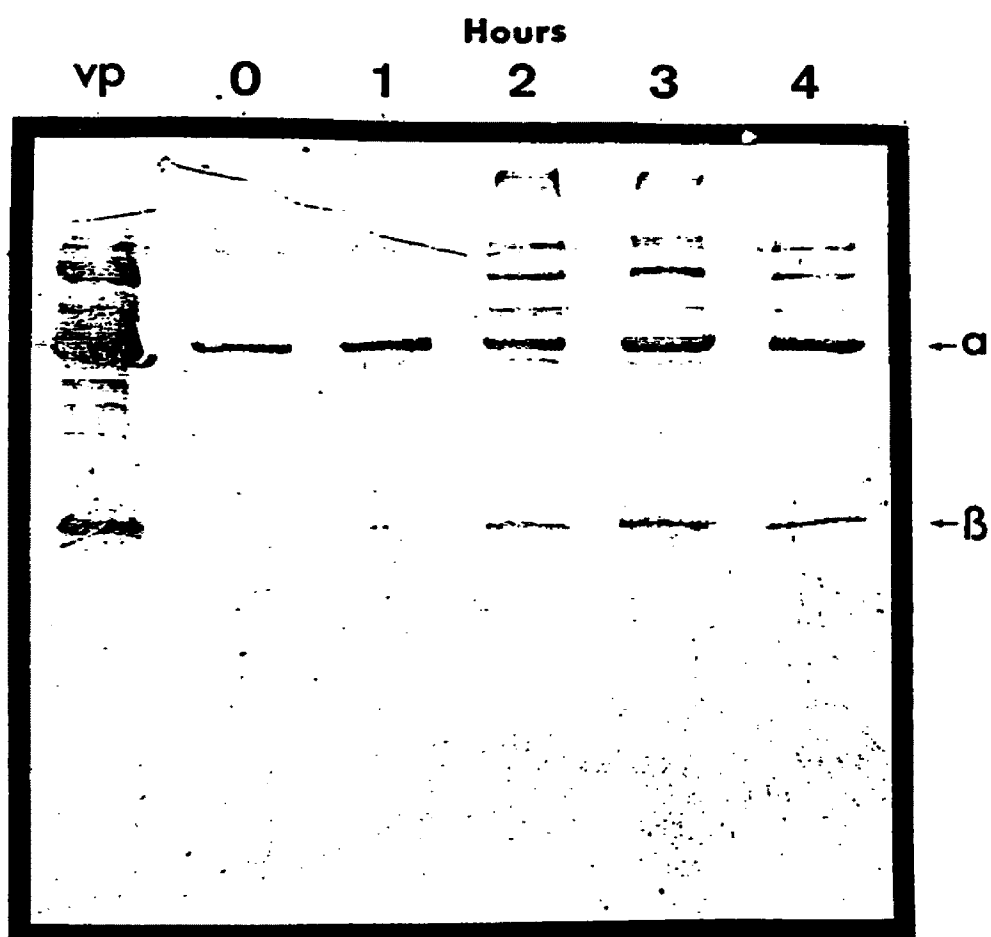


6.CHANGES IN COMPARTMENTATION OF PROTEINASE DURING HEAT STRESS

D. discoideum amoebae stressed at 40°C for designated time intervals. The cells were harvested and further resolved into the cytosolic fraction and vacuolar fraction using the same methods of subcellular fractionation. Previous studies (37) had shown that proteinase activity in the cytosolic compartment increased from 27% to 55% of total proteinase activity after 105 minutes of heat treatments. The result in Figure 14 supported the view that vacuolar proteinase was released from vacuoles into cytosol during heat stress. The data in this Figure also suggested that cytosolic proteinases, CP1 and CP2, did not increase in amount during heat stress. Other studies (20) have shown that heat stress also promoted a loss in the level and activity of inhibitor in the cytosolic fraction.

Figure 14. Changes of Proteinase Compartment in the Cytosolic Fraction during Heat Stress.

Cells were heat stressed at 40°C for various time periods and harvested. Cytosolic fractions (50 µg each) were recovered and resolved by 15% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The blot was probed with 1/100 dilution of anti-Pr1-HF serum. Lane VP, sample of vacuolar proteinase. Lane 0, control sample (Cell maintained at 23°C). Lane 1, cells incubated at 40°C for 1 hour; 2, 2 hours; 3, 3 hours; 4, 4 hours.



7. THE SECRETION OF PROTEINASE DURING DIFFERENTIATION

Finn's results (26) suggested that Pr1 was secreted during differentiation. The result in Figure 15 indicated that only VP was secreted during differentiation. In agreement with earlier studies (26), the detection of latent CBZ-Lys-ONp esterase activity in extracellular differentiation buffer suggested that the enzyme was secreted in a form in which essential sulfhydryls had been oxidized. Neither CP nor inhibitor were secreted.

8. STUDIES OF AXENICALLY CULTURED CELLS

The mutant strain Ax-3 of D. discoideum were cultured both axenically (in HL-5 medium) and with bacteria (E. coli). The proteinase activities present in whole cells extracts of cells cultured in both ways were chromatographed separately on DEAE-cellulose columns. Figure 16A shows that only one peak of esterase activity was resolved from the extract of axenically grown cells. This peak eluted at a salt concentration of approximately 0.23 M NaCl. Figure 16B shows that two major peaks of esterase activity were resolved from the extract of bacterial grown cells. The first peak of esterase activity at the salt concentration of 0.23 M NaCl corresponded to the peak resolved from the axenic grown cells. The second peak of esterase activity resolved from a salt concentration of approximately 0.3 M NaCl corresponded to vacuolar proteinase (VP). It is suggested that vacuolar proteinase (VP) could be induced by E. coli.

Figure 15. Secretion of Proteinase during Differentiation.

The sample preparation in lane 2 was the extracellular fluid collected after 16 hours of differentiation. Lane 1, the vacuolar proteinase, and lane 3, the cytosolic proteinase, were resolved by 12.5% SDS-PAGE and electrophoretically transferred onto a nitrocellular membrane. The blot was probed with 1/100 dilution of anti-Pr1 serum.

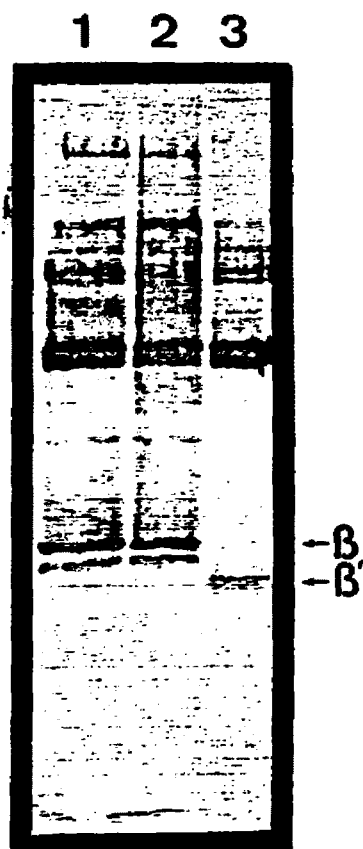
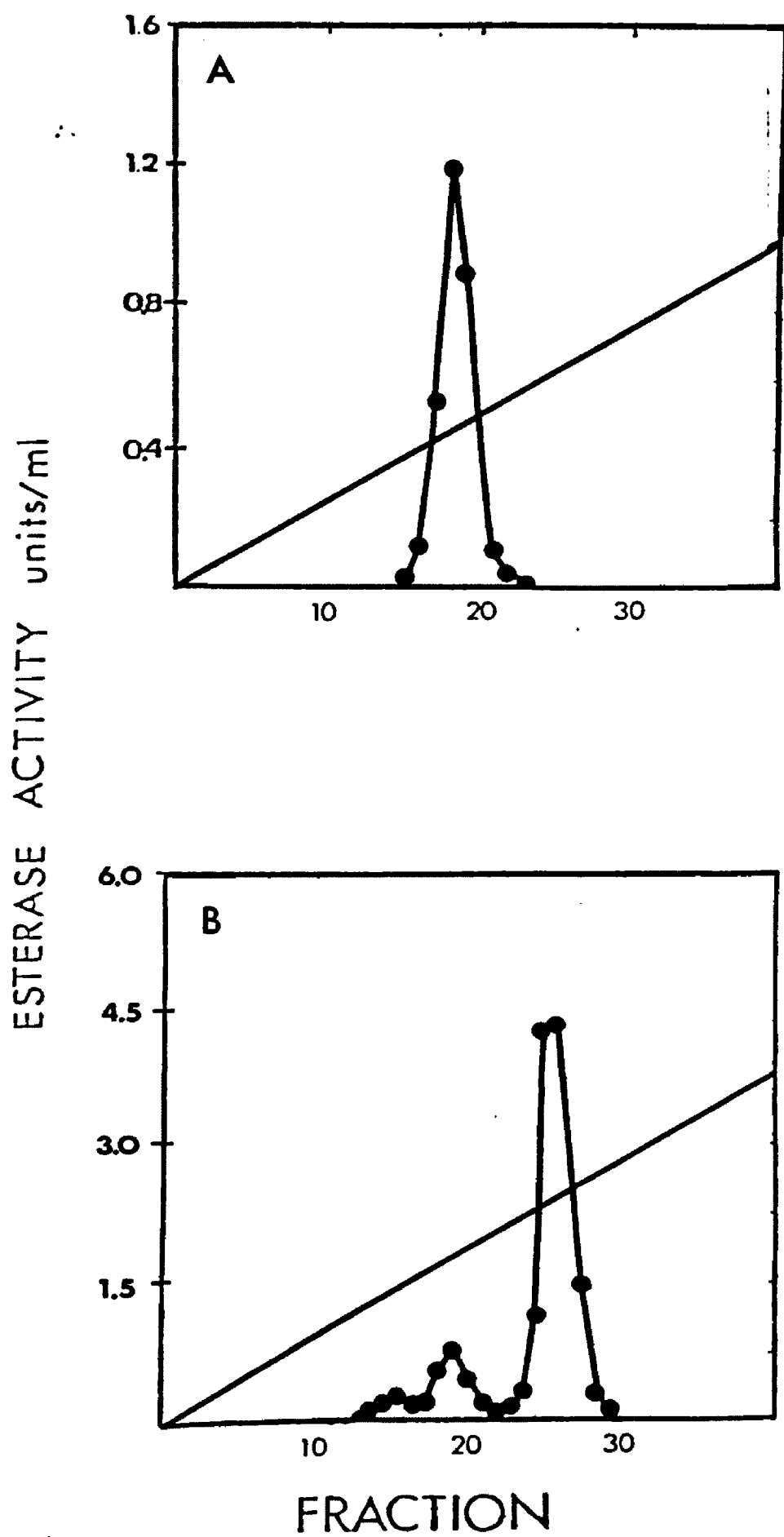


Figure 16A. DEAE-Cellulose Chromatography of a Crude Extract from Ax-3 Cells grown on HL-5 Medium.

D. discoideum Ax-3 strain cells grown on HL-5 medium were harvested at exponential growth phase (1.5×10^9 cells total). Washed cells were suspended in 1 ml of 10 mM tricine (pH 8.0) and stored at -60°C . The sample of the crude extract from the cell suspension was chromatographed on DEAE-cellulose column (1.5 x 12 cm). The column was developed with a 400 ml and linear gradient of NaCl containing 10 mM tricine (pH 8.0) and 5 mM DTT, and 5 ml fractions were collected. Fractions were assayed in the 0.2 M NaAc (pH 5.5) for esterase activity.

Figure 16B. DEAE-Cellulose Chromatography of a Crude Extract from Cells grown in Association with Bacteria.

Stationary phase amoebae of D. discoideum (1.5×10^9 cells total), grown in association with E. coli, were harvested and washed free of bacteria. The bacteria free amoebae were suspended in 10 mM tricine (pH 8.0) and stored at -60°C . The methods of DEAE-cellulose chromatography were developed in the same way as described in figure 16A.



DISCUSSION

1. The Proteolytic System of Cellular Slime Molds

A. Fractionation of vacuolar and cytosolic proteinases

The subcellular fractionation was first developed by de Duve, Palade and Clande (The Nobel Prize co-winners in 1974 for their work on subcellular fractionation). They showed that cells in a purified population could be disrupted into a cell homogenate preserving the integrity of organelles. The subcellular organelles could be separated by differential centrifugation, non-equilibrium or equilibrium density gradient centrifugation (13, 33). During differential centrifugation, the cell homogenate could be repeatedly centrifuged at progressively higher speeds in order to fractionate progressively smaller subcellular components. At a speed of 1,000 xg for 5 minutes, large components, such as nuclei and unbroken cells, were sedimented to form a pellet at the bottom of the centrifuge tube. At 15,000 xg for 20 minutes, plasma membrane, peroxisomes, vacuoles, mitochondria and lysosomes were pelleted. At 80,000 xg for 1 hour, microsomes and small vesicles were pelleted. The supernatant contained ribosomes and soluble cytosolic proteins (33).

A similar subcellular fractionation procedure was used in the present study of the proteolytic system of cellular slime mold, Dictyostelium discoideum. The proteolytic activities

attributable to aspartic (10) and cysteine proteinases (19) were previously shown to be predominantly in the 15,000 xg pellet. This fraction was designated as the vacuolar fraction. Studies by Gustafson etal (19) also suggested that the cytosolic fraction might contain masked proteinases that were different from the vacuolar enzymes. This view was supported by the results obtained in the present studies.

The studies presented in this thesis demonstrated that the vacuolar fraction of vegetative amoebae contained at least two free, active cysteine proteinases, whereas the cytosolic fraction contained two cryptic cysteine proteinase-inhibitor complexes. A major vacuolar cysteine proteinase and two cytosolic cysteine proteinases were partially purified and characterized. The latter two enzymes were co-purified.

B. The Purification of Unmasked Cytosolic Proteinases by a TCA.NaAc-treatment

Previous studies (26) had shown that two agents (i.e., TCA, SDS) could unmask proteinase activity from proteinase-inhibitor complexes. In other investigations (19), it was shown that the TCA.NaAc promoted an apparent reversible dissociation of vacuolar proteinase inhibitor complex (VPI) into free proteinase (VP) and free inhibitor (I). After unmasking in 1.3 M TCA.NaAc and subsequent dialysis to remove the chaotrope, VP and I reassociated. This reassociation

resulted in masking of the esterase activity of the proteinase - with detection of activity again requiring a high concentration of TCA.NaAc in the assay buffer. Based on these results, it was concluded that inhibitor was not degraded by vacuolar proteinase in the presence of TCA.NaAc at pH 5.5 (19).

However, cytosolic proteinases had the capability to degrade inhibitor when proteinase-inhibitor complexes were incubated in the presence of a high concentrations of TCA.NaAc buffer at pH 5.5. Apparently, the TCA.NaAc altered the conformation of cytosolic proteinases or inhibitor, so as to promote inhibitor degradation. It appeared that this was the only significant difference in catalytic properties between cytosolic and vacuolar proteinases. In proteinase purification studies, the unusual TCA.NaAc-treatment was used to prepare free cytosolic proteinases from cytosolic proteinase-inhibitor complexes.

C. Comparison of Purified Proteinase 1, Vacuolar Proteinase and Cytosolic Proteinases

Previous studies had described the isolation of a major cysteine proteinase referred to as Proteinase 1 from whole cell extracts of *D. discoideum* (12, 17). Proteinase 1 was distinguished from the other *D. discoideum* proteinases by its high affinity for protamine sulfate. In purification studies this proteinase was precipitated as a complex with protamine

sulfate. Subsequent studies (18) suggested that this property was attributable to the high content of phosphodiester-linked N-acetylglucosamine-1-phosphate residues in Proteinase 1.

One of the objectives of the present studies was to compare some of the properties of Pr1, VP, CP1 and CP2. The data collected indicated that they shared several distinctive properties; a) they could be precipitated with protamine sulfate; b) they bound tightly to DEAE-cellulose; c) CBZ-Lys-ONp could be used as the ester substrate for all proteinases; d) oxidized proteinases could be activated by treatment with dithiothreitol; and e) they combined poorly with anionic dyes (e.g., Coomassie Blue) but they reacted well with the cationic dye, Stainsall.

Based on the results in Figure 5, an ND-PAGE gel electrophoresis study, vacuolar proteinase yielded one major band. It corresponded to the upper band observed with original preparations of Pr1 (26). A lower band, corresponding to the lower band of Pr1, was obtained when vacuolar proteinase was mixed with free inhibitor. Apparently, the lower band was derived from the upper band by adding free inhibitor. Hence, the lower band was a proteinase-inhibitor complex. These results indicated that the Proteinase 1 preparation purified by Gustafson et al (16) was a mixture of vacuolar proteinase and vacuolar proteinase-inhibitor complex, and the VP was an

inhibitor-free proteinase.

In addition, based on the results of the ND-PAGE gel electrophoresis studies in Figure 6, VP, CP1, CP2 and each of their proteinase-inhibitor complexes had a unique electrophoretic property. These results demonstrated that VP, CP1 and CP2 were different cysteine proteinase species.

According to the results of SDS-PAGE studies in Figure 7 and 8, and immunoblot studies in Figure 11 and 12, VP, CP1 and CP2 each had a different small molecular weight subunit. These results also suggested that VP, CP1 and CP2 were different proteinase species. However, the fact that they shared a common subunit suggested that they might be products of related genes.

2. The mechanism of masking

In previous studies (20), a heat-stable cysteine proteinase inhibitor was purified from the cytosolic fraction of vegetative amoebae. The studies presented in this thesis have demonstrated that the cytosolic proteinase (CP1 and CP2) activities were masked by this cytosolic proteinase inhibitor. The vacuolar proteinase activity could also be masked by the inhibitor.

In many mammalian cysteine proteinase systems (21-24), the activities of cysteine proteinase have been suggested to be

regulated by the formation of protein mixed disulfides, which have a dramatic effect on proteinase catalytic activities. Generally, most inhibitors bind to proteinases at sites separate from catalytic sites. Inhibition apparently results from inhibitor-induced conformational changes in proteinases (35).

The study presented in Figure 4 suggested that the inhibitor might alter the quaternary structure of the proteinase. The masked forms of cytosolic proteinases were bigger in size than the unmasked forms based on comparison of the relative elutions during Sephadex G-200 chromatography. It was also possible that the differences in size between masked and unmasked forms might be due to the binding of the inhibitor.

In Figure 5 and 6, the unmasked native proteinase species had slower mobilities than their masked forms although the masked forms had greater molecular weight. However, ND-PAGE gel electrophoresis separates native proteins on the basis of both size and charge. Apparently, more negative charges were exposed on the surfaces of proteinase-inhibitor complexes than on free proteinases. These results might also support the view as described previously that the quaternary structure of proteinase was changed and more negative charges on the surface due to the interaction with inhibitor.

3. The Physiological function of Cytosolic Proteinases and Inhibitor

A. The physiological relationship between cytosolic proteinase and inhibitor

According to the results of subcellular fractionation studies (Table 2), cytosolic proteinases (CP1 and CP2) were masked by the cytosolic inhibitor. As discussed in the introduction, the presence of cytosolic inhibitors have been demonstrated in a variety tissue systems(35). One proposed function of the inhibitor was to prevent inappropriate proteolysis if proteinase was inadvertently released from the vacuolar compartment.

Based on the present studies it did not appear that the inhibitor was serving this kind of protective function in the amoebae of cellular slime mold. First, the cytosolic proteinases were different proteinase species than the vacuolar proteinase. Apparently, they were not released from vacuoles. Second, previous studies (37) showed that the loss of inhibitor activities accompanied by the decrease of inhibitor protein was observed during heat stress at 40°C. It appeared that inhibitor was degraded during heat stress. In addition, the results in Figure 12 showed that vacuolar proteinase was released from vacuoles into cytosol during heat stress. Apparently, the inhibitor did not serve any protective function related to a stress response.

Why *D. discoideum* amoebae would have evolved a potential to destroy the inhibitor under heat stress condition where a large amount of vacuolar proteinase was being released into cytosol is unclear. Perhaps the proteolytic activity of vacuolar proteinase was needed to help in degrading denatured or abnormal proteins generated by heat stress. It is possible that cytosolic proteinases also function in degrading some cytosolic proteins under some stress condition. Inhibitor could function to regulate both the activity of cytosolic proteinases and the activity of vacuolar proteinases released in the cytosol during different kind stresses.

An additional possibility was that cytosolic proteinases were inducible under a different stress condition. Because the cellular slime mold was supposed to live in soils of deciduous woods (33), the cytosolic proteinases might be induced by the natural seasonal changes in order to degrade abnormal cytosolic proteins for the slime mold to survive. The incubation condition at laboratory might also be a some kind stress condition, so the cytosolic proteinases were produced.

B. The physiological function of cytosolic proteinases effected by intracellular pH

It has been hypothesized that the intracellular pH plays a role in the selection of a differentiation pathway of the cellular slime molds (31). Amoebae cells expend energy to

control the pH of distinct subcellular compartments at differing values. The cytosolic pH of vegetative amoebae is approximately 7.19 (47). Changes in the cytosolic pH have been implicated in regulation of cellular metabolism, mobility, the cell cycle, and of the activation and fate of cells during development (31). The cytosolic acidification promotes the differentiation of the amoebae into pre-stalk cells, while cytosolic alkalization favors the pre-spore pathway (31). An experiment (47) showed that the cytosolic pH of amoebae could drop to 5.6 when cells were incubated with weak acids (i.e., acetic acid) or diethylstilbestrol (a plasma membrane proton translocating ATPase inhibitor), and these acidified amoebae would differentiate into pre-stalk cells after aggregation. It is possible that at pH 5.6 the cytosolic proteinase-inhibitor complexes might be dissociated and the inhibitor degraded. The activated cytosolic proteinases might serve as regulatory enzymes during the differentiation of the amoebae into pre-stalk cells.

4. The Significance Glycosylation of Proteinase

A. Significance of glycosylation of lysosome proteins

All lysosomal enzymes and most secretory proteins, including the immunoglobulins, are glycoproteins. The glycosylation apparently serves in regulating the trafficking of specific proteins between cellular organelles or between cellular membranes. Previously most evidence suggested that

glycoproteins were exclusively localized to the surface or within the lumens of intracellular organelles (e.g., lysosomes, Golgi, and endoplasmic reticulum) (33).

Lysosomal enzymes containing N-linked sugar residues are synthesized on ribosomes of the endoplasmic reticulum. The initial N-linked glycosylation occurs on nascent peptide during translation, and further processing and modification continues in the Golgi apparatus. The sugar residues then serve to route the enzyme to its final destination, serving as a "ticket" recognized by the sorting apparatus (33).

B. Significance of glycoproteins in cytosol

The cytosol consist of all of the space outside the cellular organelles and generally represents 50% to 60% of total cell volume. The amount of any particular protein in the cytosol depends on the balance between the rate at which it is synthesized and the rate at which it is degraded. Some proteins are rapidly changed in their concentrations in response to regulatory signals. It has been suggested that the presence of carbohydrate covalently bound to certain proteins altered their rates of degradation (48).

It is not clear how glycoproteins would leave the ER-Golgi-lysosomal system and enter the cytosol. The studies presented in this thesis have suggested that vacuolar and

cytosolic proteinases shared a common pattern of phosphoglycosylation but different compartmentations. The pathway of assembly of O-phosphodiester-linked sugars is not clear, however, the common occurrence of phosphodiester linked GlcNAc-P residues in VP, CP1 and CP2 suggested that these enzymes were processed in the same compartment. It is possible that these proteinases may be synthesized in the cytosol and then transported to the proper compartment post-translationally. Post translational insertion of proteins into organelles is common and has been shown for some mitochondrial enzymes (36).

Recently, other cytosolic glycoproteins modified by O-linkages of single residues of N-acetylglucosamine (GlcNAc) have also been found in lymphocytes (38), rat liver cells (39), and human erythrocytes (40). In addition, O-linked GlcNAc glycoproteins have also been found on the cytoplasmic surface of the nuclear pore complex from the human erythrocytes (41).

C. The physiological function of O-linked and O-phosphodiester-linked glycoproteins in cytosol

The role that O-linked glycosylation plays in the functions of cytosolic glycoproteins remains unknown, although several hypotheses have recently been proposed. For example, the enrichment of O-GlcNAc-bearing glycoproteins in the nucleus

have suggested that O-GlcNAc serves as a nuclear transport signal (42). Blocking of the carbohydrate sites on nuclear pore complex glycoproteins with wheat germ agglutinin was shown to inhibit the transport of nucleoplasmic across the nuclear envelope (43). Alternatively, it is also possible that cytosolic O-linked glycosylation is important for the proper assembly of certain multimeric protein structures, such as the nuclear pore complex or the cytoskeletal associated protein (44) which may attach the cytoskeleton to the plasma membrane by serving as a bridge protein between spectrin, actin, and the cytosolic portion of glycophorin.

In the studies of this thesis, it is assumed that O-phosphodiester linked N-acetylglucosamine-1-phosphate residues of D. discoideum cysteine proteinases might provide an important mechanism for regulating the degradation rates of cytosolic proteinases in the cytosol of amoebae.

5. The Regulation of Cysteine Proteinase Synthesis by the Bacterial Factor

According to the results in Figure 15, the vacuolar proteinase was secreted during differentiation. The results in the axenic strain Ax-3 studies in Figure 16, the axenically grown slime mold could not produce phosphoglycosylated vacuolar proteinase (VP). However, the bacterial (Gram negative bacteria E. coli) grown slime mold, strain Ax-3, could produced vacuolar

proteinase (VP). Apparently, the VP could be induced by a cysteine proteinase inducing factor derived from bacteria.

Recently, peptidoglycan has been suggested as the cysteine proteinase inducing factor (46). An alternative observation suggested that the endotoxin, lipopolysaccharide, could modulate the expression of proteinase inhibitor in human monocytes and macrophages (49). These views suggest that both peptidoglycan and lipopolysaccharide might be involved in regulating cysteine proteinase and inhibitor in the cellular slime mold.

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